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TRANSFER OF ARTERIAL HYPERTENSION BY SPLENIC CELLS FROM DOCA-SALT HYPERTENSIVE AND RENAL HYPERTENSIVE RATS TO NORMOTENSIVE RECIPIENTS

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Olsen F Transfer of arterial hypertension by splenic cells from DOCA salt hypertensive and renal hypertensive rats to normotensive recipients Acta path. microbiol scand Sect. C 88 1–5 1980

Arterial hypertension was transferred from DOCA salt hypertensive and renal hypertensive rats to normotensive rats by intravenous injection of splenic cells Thirteen normotensive recipients were injected intravenously with splenic cells from the hypertensive donors Eleven developed arterial hypertension (85%) that is with a systolic blood pressure exceeding 140 mm Hg Three of the recipients developed hypertensive levels up to 155–160 mm Hg which was almost up to the levels in the donors The increase of the blood pressure in the recipients was significant when compared to controls injected intravenously with splenic cells from normotensive donors ($p < 0.001$) Skin tests performed by intracutaneous injection of homogenized common carotid arteries in half of the recipients showed positive reactions 24 hours after the injection Microscopical examination of heart and kidney from the other half demonstrated mononuclear infiltration into arterial and arte[illegible] etudative changes in these walls Due [illegible] narrowed The hypothesis is advanced th[illegible] of a transferred delayed hypersensitivi[illegible] reaction caused insudation of plasma con[illegible] to the arterial walls narrowing of their lumina and an increased peripheral resistance to the blood flow so that arterial hypertension developed

Key words Arterial hypertension transfer splenic cells

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In the last ten years many published observations have indicated a correlation between arterial hyper tension and immunological factors Interest in this problem seems to be increasing (*The Lancet* 1978) The possible pathogenetic role of these factors in arterial hypertension is still unsolved Hypersensiti vity of the delayed type (DHS) directed against components in arterial and arteriolar walls seems to develop in experimental cases of acute arterial hypertension (*Olsen* 1970 1971 *Svendsen* similar hypersensitivity has been dem patients with essential hypertension (*Olsen & Rasmussen* 1977) Finally it has been suggested that the chronic phase of experimental DOCA-salt hypertension and renal hypertension in mice is thymus dependent (*Svendsen* 1976 1977)

The aim of the present study was to investigate whether it is possible to transfer arterial hyperten sion by splenic cells from hypertensive rats to normotensive rats

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The aim of the present study was to investigate whether it is possible to transfer arterial hypertension by splenic cells from hypertensive rats to normotensive rats

MATERIAL AND METHODS

Animals Genetically identical rats F1 (SA × BN) hybrids of both sexes were used Weight about 225 g

Anaesthesia A solution of amylobarbitone sodium (Amytal®) 25 mg/ml was injected intraperitoneally in a dose of about ½ ml per 100 g body weight

Measurement of the blood pressure was performed by a noninvasive method applied on the tail of unanaesthetized animals The principle of this method is based upon a photoelectric device described in detail by *Palbøl & Henningsen* (1979) The precision of the method was ± 2% when compared to a simultaneous invasive measurement performed with a needle placed in the common carotid artery

A Hypertensive Donors of Splenic Cells

Group I Desoxycorticosterone acetate (DOCA) salt hypertension was induced in 9 rats by subcutaneous injections of Percorten M® in a weekly dose of 12½–25 mg and 1% salt in the drinking water The rats were used as splenic cell donors when they had been hypertensive for about three months meaning that their blood pressure had exceeded 140 mm Hg for at least three months At this time the four rats with the highest hypertensive levels were killed with ether and the spleen was removed immediately thereafter The pooled splenic cells were washed three times in Eagle s minimum essential medium and finally suspended in about 5 ml of this medium to which was added rat serum protein (10%) penicillin (100 IU/ml) and streptomycin (100 μg/ml) About 95% of the splenic lymphocytes were alive at the time of the intravenous injection into the normotensive recipients

Group II One week after unilateral nephrectomy had been performed under amylobarbitone sodium anaesthesia *renal hypertension* was induced in six rats by partial infarction of the remaining kidney by ligation of a branch of the renal artery These rats were used as splenic cell donors when they had been hypertensive for about three months At this time the four rats with the highest hypertensive levels were used as splenic cell donors in a similar way as described above for the DOCA salt hypertensive donors of splenic cells About 95% of the splenic lymphocytes were alive at the time of the intravenous injection into the normotensive recipients

B Normotensive Recipients of Splenic Cells from the Hypertensive Donors

Group III Recipients of splenic cells from DOCA salt hypertensive donors Seven rats were lightly anaesthetized with amylobarbitone sodium Thereafter a polyethylene catheter was placed in the femoral vein and a total of about 180 million splenic lymphocytes were injected intravenously into each recipient

Group IV Recipients of splenic cells from renal hypertensive donors Six rats were treated as mentioned above for the recipients in group III and each was injected intravenously with about 170 million splenic lymphocytes

C Control Rats

Group V Seven rats were lightly anaesthetized with amylobarbitone sodium and a polyethylene catheter was placed in the femoral vein as described above From four normotensive rats 175 million splenic lymphocytes about 95% of which were alive were injected intravenously into each of the seven normotensive recipients

Group VI The hearts and kidneys from three untreated normotensive controls were examined histologically

D Skin Test

Six common carotid arteries were removed from three rats genetically identical with the rats of groups III and V The carotid arteries were cleaned for blood and thereafter homogenized by homogenizer pestles for about ten minutes at 4 °C The homogenate was suspended in about 5 ml of Hanks buffered salt solution and stored overnight at 4 °C The homogenate was injected intracutaneously in a dose of 0 1 ml into the back of the rats of group III when the blood pressure measurements in these rats had been discontinued i e about six months after the transfer of splenic cells from DOCA salt hypertensive rats The result of the skin test was read after 24 hours (*Brændstrup et al* 1975) The skin test on the control rats in group V was performed about six months after the transfer of splenic cells when their blood pressure measurements had been discontinued The result was read after 24 hours and immediately thereafter the rats were killed

Microscopical preparations The heart and kidney from the rats of groups IV V and VI were fixed in 4% formalin embedded in paraffin cut into sections five microns thick and stained with haematoxylin eosin A total of 120 sections from each heart and kidney were examined under the light microscope

RESULTS

A Hypertensive Donors of Splenic Cells

Group I (Rats treated with injections of DOCA and 1% salt in the drinking water) The hypertensive effect of the treatment with DOCA salt is shown in Fig 1 The rats developed arterial hypertension about 3 months after the treatment was started and remained hypertensive during the next three months At that time they were sacrificed the spleen was removed and the splenic cells were transferred to normotensive recipients The mean blood pressure in the four donor rats was 160 mm Hg

Group II (renal hypertensive rats) The hypertensive effect of the partial infarction of the kidney is illustrated in Fig 1 which shows that the rats developed arterial hypertension about 2 months after ligation of a branch of the renal artery The hypertension was maintained during the next three months At that time the rats were sacrificed the spleen was removed and the splenic cells were transferred to normotensive recipients The mean blood pressure in the four donor rats was 153 mm Hg

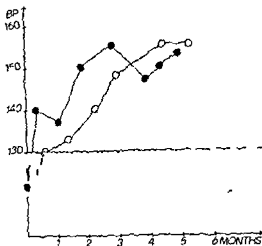


Fig 1 The development of arterial hypertension in the donor rats of splenic cells. Abscissa Months after institution of treatment with DOCA-salt (O) and after partial infarction *of one kidney and contralateral* nephrectomy (●) Ordinate Mean blood pressure

The hatched area indicates the blood pressure levels in normotensive control rats

B Recipients of Splenic Cells from Hypertensive Donors

Group III (Recipients of splenic cells from DOCA-salt hypertensive donors) The effect on the blood pressure of transferring splenic cells from the DOCA-salt hypertensive donors to the *normotensive* recipients is presented to *Fig 2 The recipients*

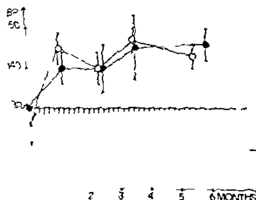


Fig The development of arterial hypertension in the recipient rats after transfer of splenic cells *Abscissa* Months after transfer of splenic cells from DOCA salt hypertensive donors (O) and renal hypertensive donors (●) *Ordinate* Mean blood pressure ± SEM

The hatched area indicates the *blood pressure* in untreated normotensive *control rats* Mean value in the control rats 125 mm Hg ± SEM

developed arterial hypertension in the course of the first month after the transfer of splenic cells and the hypertension persisted throughout the observation period which was about six months The mean blood pressure during the observation period ranged from 140 to 148 mm Hg The highest individual levels in the recipient rats were 160 mm Hg in one rat and 150 mm Hg in another two One hypertensive rat died in the middle of the observation period Five of the remaining six rats were hypertensive throughout the observation period i e their blood pressure exceeded 140 mm Hg The increase in blood pressure after the transfer of splenic cells to the normotensive recipients was significant in comparison to the recipients blood pressure before the transfer of the splenic cells and to the control rats in group V ($p < 0.001$ u test)

Group IV (Recipients of *splenic cells from renal* hypertensive donors) The effect on the blood pressure of transferring splenic cells from the renal hypertensive donors to the normotensive recipients is set out in Fig 2 The blood pressure in the recipients increased to borderline hypertensive levels in the course of the first three months after the transfer of splenic cells During the next three months the blood pressure increased further to significantly hypertensive levels. *The mean blood pressure during* the total observation period ranged *from 140 to 147 mm Hg The highest individual blood pressures in the recipient rats were 160 mm Hg in one rat 155 mm Hg* and 150 mm Hg in two and 145 mm Hg in another two By definition five *of the six recipients developed arterial hypertension*

The increase in blood pressure after the transfer *of splenic cells* to the normotensive recipients was significant in comparison with the *recipients blood* pressure before the transfer of splenic cells and *with* the control rats in group V ($p < 0.001$ u test)

C Control Rats

Group V (Recipients *of splenic* cells from *normotensive donors*) Transfer of *splenic cells from* normotensive rats to normotensive recipients did not change the *recipients blood pressure They* remained normotensive at the same *level as before* the transfer of splenic cells throughout *the six* months observation period

D Skin Test

The results of *skin tests* performed with homogenized *common carotid* arteries on the rats in group III *are listed in Table 1* Intracutaneous *injection of the* homogenized arterial vessels resulted in an unmistakable skin infiltration after 24 hours

No infiltration was found in the group V rats

Anaesthesia A solution of amylobarbitone sodium (Amytal®) 25 mg/ml was injected intraperitoneally in a dose of about ½ ml per 100 g body weight

Measurement of the blood pressure was performed by a noninvasive method applied on the tail of unanaesthetized animals The principle of this method is based upon a photoelectric device described in detail by *Palbøl & Henningsen* (1979) The precision of the method was ± 2% when compared to a simultaneous invasive measurement performed with a needle placed in the common carotid artery

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Group V Seven rats were lightly anaesthetized with amylobarbitone sodium and a polyethylene catheter was placed in the femoral vein as described above From four normotensive rats 175 million splenic lymphocytes, about 95% of which were alive, were injected intravenously into each of the seven normotensive recipients

Group VI The hearts and kidneys from three untreated normotensive controls were examined histologically

D Skin Test

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Microscopical preparations The heart and kidney from the rats of groups IV, V and VI were fixed in 4% formalin embedded in paraffin cut into sections five microns thick, and stained with haematoxylin-eosin A total of 120 sections from each heart and kidney were examined under the light microscope

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Group II (renal hypertensive rats) The hypertensive effect of the partial infarction of the kidney is illustrated in Fig 1 which shows that the rats developed arterial hypertension about 2 months after ligation of a branch of the renal artery The hypertension was maintained during the next three months At that time the rats were sacrificed, the spleen was removed and the splenic cells were transferred to normotensive recipients The mean blood pressure in the four donor rats was 153 mm Hg

III and negative in the controls (group V) and the histological examinations of small arteries and arterioles demonstrated findings which could accord with a DHS reaction against these vessels Thus the results were in agreement with the hypothesis that a DHS-response against the walls of arteries and arterioles could be responsible for the positive transfer of arterial hypertension from hypertensive animals to normotensive recipients

The histological examinations of the kidneys and the hearts demonstrated many arterial vessels with thickened walls and consequently narrowed lumina This morphological change of the arterial walls was apparently caused by exudation of plasma into the vessel walls rather than by hypertrophy of the tunica media The exudative changes of the arterial walls could be secondary to the arterial hypertension but it seems unlikely that the hypertension *alone* was responsible for the exudative changes because the hypertension was mild and it had continued for several months Possibly, hypertrophy of the tunica media would have been the natural reaction of the arterial walls to this mild and chronic arterial hypertension (*Giese* 1966)

The infiltration of mononuclear cells into the arterial walls found both in walls with exudative changes and in walls which appeared normal in the light microscope may be interpretated as a DHS reaction against components of the vessel walls The observation that mononuclear cells infiltrated apparently normal arterial walls could indicate that an immunological reaction of the delayed type represents a primary stage in the morphological change of the arterial vessels

In the light of the present and previous results (*Olsen* 1970 1971 *Svendsen* 1973 1975 1976) the following hypothesis is advanced in explanation of the transfer of arterial hypertension by splenic cells from DOCA salt hypertensive and renal hypertensive donors to normotensive recipients An immunological reaction of the delayed type directed against the arterial walls is transferred by the splenic cells from the hypertensive donors to the normotensive recipients This transferred hypersensitivity may cause an increased permeability of the arterial walls to plasma components resulting in a thickening of the arterial walls and consequently narrowed lumina When the lumina of small arteries and arterioles are narrowed peripheral resistance to blood flow increases and arterial hypertension results

The mentioned hypothesis may explain the development of some cases of essential hypertension in patients The observations of DHS-reactions against components of arterial walls in patients suffering from *borderline* essential hypertension (*Olsen & Rasmussen* 1977) is in agreement with this hypothesis

The author is very thankful to the *University Institute for Experimental Immunology* Copenhagen for supplying the splenic cells

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The Lancet Immunogenetics and essential hypertension (Editorial) *II* 409–410 1978

TABLE 1 *Cutaneous Infiltration Measured in mm Found in the Rats in Groups III and V, 24 Hours after the Intracutaneous Injection of Homogenized Common Carotid Arteries*

Group III	
Rats	Cutaneous infiltration (in mm)
No 1	8
No 2	9
No 3	10
No 4	12
No 5	10
No 6	2

Group V	
Rats	Cutaneous infiltration (in mm)
No 1	0
No 2	0
No 3	0
No 4	0
No 5	0
No 6	0
No 7	0

after intracutaneous injection of homogenized common carotid arteries (Table 1)

Microscopical examinations The histological preparations of the kidneys and the hearts from the rats of group IV showed many small arteries and arterioles with thickened walls and narrowed lumina The ratio lumen wall was decreased in relation to normal arterial vessels (group VI) In most cases the cause of this decreased ratio seemed to be exudative changes in the vessel walls and to a lesser degree hypertrophy of the tunica media Thus an increased insudation of plasma seemed to have taken place into these vessel walls which also was supported by the finding of red blood cells deposited in the tunica media of some arterial vessels Furthermore mononuclear cells were found to infiltrate into many arterial walls This mononuclear infiltration was observed both into vessels with greatly thickened walls and vessel walls which were morphologically normal under the light microscope The number of infiltrating mononuclear cells ranged from 3 4 to numerous

None of the rats in group V showed thickening of arterial walls in the kidney or the heart No red blood cells or mononuclear cells were found in the arterial walls The histological findings in this group were like those in all three normal untreated rats (group VI)

DISCUSSION

The results show that it was possible to transfer arterial hypertension from DOCA salt hypertensive and renal hypertensive rats to normotensive rats by splenic cells The results of the transfer experiments from renal hypertensive rats to normotensive recipients are in agreement with those reported by *Okuda & Grollman* (1967) who succeeded in transferring renal hypertension to normotensive recipients by lymph node cells

A new aspect in the present study is the positive transfer of DOCA-salt hypertension to normotensive recipients by splenic cells The hypertensive mean value in groups III and IV was unquestionable, but the hypertension was of the benign type The mean value did not exceed 148 mm Hg during an observation period of six months after the transfer of splenic cells While the hypertensive mean value in the two groups did not exceed 148 mm Hg some individuals of the two groups developed hypertensive blood pressures up to 155–160 mm Hg These levels were nearly identical to those in the donor rats *Okuda & Grollman* (1967) were unable to elucidate the mechanism by which the transfer experiments had succeeded using Loomis hypertensive rats as donors of splenic cells In their opinion the immunological mechamism was a consequence of the elimination of the renal capacity to produce a postulated humoral principle essential for the maintenance of the normotensive state but no observations could support this view

The present results show that it is possible to transfer *two very different types* of arterial hypertension from hypertensive animals to normotensive recipients by splenic cells These positive transfer experiments suggest that in spite of the fact that the two types of arterial hypertension are different in origin they have a common immunopathogenesis in the late phase of the hypertension Previous results (*Olsen* 1970 1971, *Svendsen* 1973 1975 1976) have demonstrated that induction of arterial hypertension in rats and mice is associated with damage of arterial vessels and the development of DHS against their own arterial walls The responsible antigen(s) of the arterial walls have not been isolated In the light of these observations the common immunological mechanism in the present experiments may be a DHS response against the walls of arteries and arterioles Therefore skin tests were performed in group III and histological examinations in group IV Skin tests using extract of common carotid arteries were positive in group

III and negative in the controls (group V) and the histological examinations of small arteries and arterioles demonstrated findings which could accord with a DHS reaction against these vessels Thus the results were in agreement with the hypothesis that a DHS-response against the walls of arteries and arterioles could be responsible for the positive transfer of arterial hypertension from hypertensive animals to normotensive recipients

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after intracutaneous injection of homogenized common carotid arteries (Table 1)

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None of the rats in group V showed thickening of arterial walls in the kidney or the heart No red blood cells or mononuclear cells were found in the arterial walls The histological findings in this group were like those in all three normal untreated rats (group VI)

DISCUSSION

The results show that it was possible to transfer arterial hypertension from DOCA salt hypertensive and renal hypertensive rats to normotensive rats by splenic cells The results of the transfer experiments from renal hypertensive rats to normotensive recipients are in agreement with those reported by *Okuda & Grollman* (1967) who succeeded in transferring renal hypertension to normotensive recipients by lymph node cells

A new aspect in the present study is the positive transfer of DOCA salt hypertension to normotensive recipients by splenic cells The hypertensive mean value in groups III and IV was unquestionable but the hypertension was of the benign type The mean value did not exceed 148 mm Hg during an observation period of six months after the transfer of splenic cells While the hypertensive mean value in the two groups did not exceed 148 mm Hg some individuals of the two groups developed hypertensive blood pressures up to 155–160 mm Hg These levels were nearly identical to those in the donor rats *Okuda & Grollman* (1967) were unable to elucidate the mechanism by which the transfer experiments had succeeded using Loomis hypertensive rats as donors of splenic cells In their opinion the immunological mechanism was a consequence of the elimination of the renal capacity to produce a postulated humoral principle essential for the maintenance of the normotensive state but no observations could support this view

The present results show that it is possible to transfer two very different types of arterial hypertension from hypertensive animals to normotensive recipients by splenic cells These positive transfer experiments suggest that in spite of the fact that the two types of arterial hypertension are different in origin they have a common immunopathogenesis in the late phase of the hypertension Previous results (*Olsen* 1970 1971 *Svendsen* 1973 1975 1976) have demonstrated that induction of arterial hypertension in rats and mice is associated with damage of arterial vessels and the development of DHS against their own arterial walls The responsible antigen(s) of the arterial walls have not been isolated In the light of these observations the common immunological mechanism in the present experiments may be a DHS response against the walls of arteries and arterioles Therefore skin tests were performed in group III and histological examinations in group IV Skin tests using extract of common carotid arteries were positive in group

MACROPHAGES AND THE SYNTHESIS OF CONNECTIVE TISSUE COMPONENTS

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1 Macrophage homogenate added to the incubation medium of granulation tissue slices decreases the incorporation of labelled proline to collagen and other proteins to a greater extent in the presence of certain prostaglandins and in oxygen atmosphere but to a lesser degree if serotonin and cyclic AMP are added 2 Rheumatoid synovial fluid connective tissue active peptide (CTAP) of Castor and an extract from CCl_4-damaged liver stimulate collagen synthesis in the presence of macrophages 3 Preparations from non treated macrophages stimulate the incorporation of glucosamine to acid mucopolysaccharides mainly hyaluronate 4 In a developing rat granuloma the formation of collagen was stimulated by homogenized macrophages and by macrophage culture medium but especially by whole macrophages in the presence of SiO_2

Key words Collagen DNA hyaluronic acid SiO_2

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This study was inspired by the finding that SiO_2 releases from macrophages a »fibrogenic factor« which stimulates protein synthesis in incubated granulation tissue slices (1) and in cell free polysomal systems (4) Since SiO_2 and related minerals presumably are not the only substances to act on macrophages in this manner we have investigated the possible analogous effects of certain drugs and endogenous materials

The role of macrophages in the early development of granuloma (3) and the influence of macrophages on the adjacent fibroblasts (23) have a bearing on the fibrotic process This encouraged us to study the effect of macrophage preparations on the synthesis of protein especially of collagen of acid mucopolysaccharides and of nucleic acids in granulation tissue Furthermore we carried out preliminary experiments on the effects of macrophages on the integrated development of granulation tissue Previously the medium of macrophages is shown to contain several connective tissue active substances affecting the synthesis of DNA and collagen (15)

MATERIALS AND METHODS

Test Systems

Incubation of granulation tissue slices Experimental granulomas were induced with viscose cellulose sponge implanted subcutaneously into rats (28) After 15–21

[illegible]

proline (TRA 82 The Radiochemical Centre Amersham Bucks U K) thymidine (TRK 300) or glucosamine (TRK 375) was added as precursor

Incubation of granuloma cells After growth for two weeks the sponge implants were harvested into cold 0 9% NaCl solution The cultivation of the granuloma cells has been described previously (2) The cells from the third to the tenth passage were used for incorporation experiments The confluent cells were trypsinized diluted 1 4 and added to cell culture tubes (Nunclon® N 1409) No exogenous proline was added except 5 μCi of ^3H proline Incubations were carried out in the presence of 10% foetal calf serum (FCS) in 5% CO_2 + 95% air ^3H Thymidine and ^3H glucosamine were used as alternative precursors

Development of granulation tissue in vivo Discs 10 mm thick and 15 mm in diameter were cut from the

TABLE 1 *Effect of the Subcellular Fractions of the Homogenized Macrophages on the Collagen Synthesis in Incubated Granulation Tissue Slices*

Macrophage fraction added	Hydroxyproline cpm per 100 mg nitrogen	Hydroxyproline cpm per µmole
None (control)	0 568	1136
500 g sediment	0 488 (+0 057)	875 (+166)
7000 g sediment	0 444 (+0 030)	761 (+158)
100 000 g sediment	0 688 (−0 153)	930 (+ 54)
100 000 g supernatant	0 511 (−0 264)	805 (−206)

Non incubated macrophages were homogenized by freezing and thawing (5×) then fractionated and the fractions preincubated separately at 37 °C for 20 h in the Dulbecco-MEM medium The granulation tissue slices were incubated in the 20 000 g supernatants of these suspensions in the presence of ^{3}H proline (1) The slices were analysed for incorporated isotope The effect of the preincubation of whole macrophages for 24 h is shown in the brackets

TABLE 2 *Effect of Various Small molecular Substances on the Macrophages in Modulating the Synthesis of Collagen and Other Proteins in Granulation Tissue Slices*

Addition	Total protein per dry wt.	Total protein per nitrogen	Hydroxyproline per dry wt.	Hydroxyproline per µmole
Control with normal macrophages	100	100	100	100
Prostaglandin $PGF_{2\alpha}$ 100 µg/ml	87	86	–	–
Prostaglandin PGE_1 100 µg/ml	77	79	58	53
Oxygen atmosphere	81	87	65	65
Serotonin 10^{-5}M	116	123	153	129
Serotonin 10^{-3}M	80	91	96	126
Cyclic AMP 50 µg/ml	101	108	107	103
Cyclic AMP 100 µg/ml	104	108	114	116

The control experiments (100 0%) were performed without additions which were made to the macrophage medium not to the final incorporation medium All experiments were carried out in duplicate

TABLE 3 *Effect of Various Macromolecular Materials on the Macrophages in Modulating the Synthesis of Collagen and Other Proteins in Granulat on Tissue Sl ces*

Addition		Synthesis of Total protein[a]	Synthesis of Hydroxyproline[a]
None (control)		100	100
Synovial fluid rheumatoid	(4)	117 5 ± 4 4	141 8 ± 4 2
None (control)		100	100
CTAP (alone)[b]	(2)	81 (70 91)	62 (43 80)
CTAP + macrophages as usual	(2)	116 (111 121)	101 (100 101)
Extract from control liver	(2)	100	100
Extract from CCl_4-damaged liver	(2)	112	122

The experiments with control macrophages taken as the reference ~ 100% ± S.E.M
Number of the experiments given in brackets
[a] cpm calculated per tissue nitrogen
[b] no macrophage preparation present.

viscose cellulose sponge, sterilized by boiling in water and pressed dry. They were then placed in well-fitting plastic cylinders with both ends open. Before implantation (four pieces per rat) the sponges were absorbed with 1.2–1.3 ml of sterile suspension of non-elicited live rat peritoneal macrophages (0.9×10^6 cells/ml) or other test material (macrophage homogenate or medium). It was ascertained that at least 45–50 % of trypan blue absorbed similarly remains in the sponge for two weeks. No signs of infection were observed in the two experimental series of *16* and *21* days. For analysis, granulomas from three rats were combined into one sample. Incorporation experiments with the granuloma slices were carried out as described above.

Additions of Biological Origin

Macrophage preparations. Rat peritoneal macrophages were collected into 0.9 % NaCl containing 10 IU/15 ml heparin. The disintegration and preparation of the 20000 *g* supernatant have been described previously (1). The test substances were added instead of SiO_2, and the final supernatant was used as medium for the incubations with *granulation tissue slices (1) or cultured granuloma* cells.

The medium for the culture of rat peritoneal macrophages was Dulbecco's modification of Eagle's medium (No 1F–017A, Flow Laboratories Ltd., Irvine, Ayrshire, Scotland), buffered with 20 mM Hepes and supplemented with penicillin and streptomycin (1). The peritoneal cells were washed twice in this medium and placed into disposable 5 ml or 10 ml plastic Nunclon cell culture bottles, $3–4 \times 10^6$ cells/ml medium. The cultures were incubated for 2 h at 37 °C in an atmosphere of 5 % CO_2 + 95 % air. The non-adherent cells were rinsed away with the medium. Fresh medium was added and the cultivation continued for two-day periods. The used media were centrifuged at 20000 *g* for 30 min and stored at −20 °C until use. Addition of serum was not necessary. Storage of the macrophage media for two months at −18 °C did not have any harmful effect on the subsequent incorporation experiments.

Synovial fluids, synovial tissue extracts and connective tissue activating peptide. Connective tissue activating peptide (CTAP) was prepared from the »buffy coat« of human blood according to *Castor & Lewis* (10). The leukocytes were lysed by freezing and thawing in 0.05 M phosphate buffer, pH 7.0, containing 0.15 M NaCl and 0.13 M 2-mercaptoethanol. The mixture was centrifuged at *17000 g for 15 min and gel-filtered in a Sephadex* G-50 column (2.8×50 cm). Elution with the phosphate buffer containing NaCl and mercaptoethanol yielded two fractions, the second of which contained the CTAP. Synovial fluid from the knee of a rheumatoid patient was diluted with a half volume of 4.5 % NaCl and treated with hyaluronidase for 5 h. The digest was fractionated on Sephadex G-200 column (2.7×40 cm) which was eluted with Aronson's buffer (5) diluted 1:20 with 0.9 % NaCl. The fractions were dialysed and lyophilized. The amounts of the fractions added to macrophages were adjusted so that each contained 207 μg protein.

CCl_4-treated rats received 75 μl CCl_4/100 g in liquid paraffin at 3 to 4-day intervals for 32 days. The livers were homogenized with an Ultra-Turrax *homogenizer* into a five-fold volume of Krebs-Ringer-Hepes medium. The suspension was centrifuged at 15000 *g* for 20 min and the supernatant added to the medium of granulation tissue slices to be incubated (1).

Analytical Methods

Uronic acid, hexosamine and nitrogen were determined as described previously (18).

Radioactive products. Collagen and other proteins containing radioactive proline were hydrolysed in 6 N HCl for 3 h and the radioactivities of total protein and hydroxyproline were determined as described (1, 16, 18). *Nucleic acids were fractionated after the principle of Schmidt & Thannhauser* (25). DNA was determined according to *Burton* (9) and RNA was measured as described by *Ceriotti* (12). Radioactive acid mucopolysaccharides were precipitated with CPC onto 0.45 μm Millipore filter and the hyaluronate fraction was obtained by dissolving the precipitate into 0.5 M hydrochloric acid as described by *Saarni & Tammi* (24). The radioactivities were measured with a Packard Liquid Scintillation Spectrometer Model 3353 with PPO-Tergitol scintillation mixture (15 g PPO, 50 mg POPOP, 600 ml toluene and 400 ml Tergitol®, Union Carbide 15-S-9, supplied by Turun Saippua Oy, Turku, Finland).

RESULTS

Synthesis of Collagen and Other Proteins

Addition of macrophage homogenate to the medium in which granulation tissue slices were to be incubated caused a marked decrease in the incorporation of labelled proline (−22.3 % in total protein and −31.4 % in collagen). The decrease is due largely to the cold proline of the macrophages, which dilutes the specific activity of the precursor proline. This was ascertained by the addition of cold proline to the medium in two concentrations, 2.87 mM and 0.287 mM. The proline pool, calculated from the radioactivity ratios observed was about 1.6 mM in granulation tissue, and of the same order of magnitude in the added macrophages. However, in the 20000 *g* supernatant of the 7000/500 *g* particles of macrophages, the proline concentration was very small, and this source of error thus insignificant.

The possible effect of the subcellular particles of macrophages on the synthesis of protein is shown in Table 1. During preincubation of the whole macrophages, material is released from the particles into the 100000 *g* supernatant to prevent the incorporation of ^3H proline into collagen. The 100000 *g* sediment seems to have a stimulating effect, which is more apparent in the synthesis of total protein (data not shown). When the 100000 *g*

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TABLE 2 *Effect of Various Small molecular Substances on the Macrophages in Modulating the Synthesis of Collagen and Other Proteins in Granulation Tissue Slices*

Addition	Total protein per dry wt.	Total protein per nitrogen	Hydroxyproline per dry wt	Hydroxyproline per µmole
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The control experiments (− 100 0%) were performed without additions which were made to the macrophage med um not to the final incorporation medium All experiments were carried out in duplicate

TABLE 3 *Effect of Various Macromolecular Materials on the Macrophages in Modulating the Synthesis of Collagen and Other Proteins in Granulation Tissue Slices*

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Synovial fluid rheumatoid	(4)	117 5 ± 4 4	141 8 ± 4 2
None (control)		100	100
CTAP (alone)[b]	(2)	81 (70 91)	62 (43 80)
CTAP + macrophages as usual	(2)	116 (111 121)	101 (100 101)
Extract from control liver	(2)	100	100
Extract from CCl_4-damaged liver	(2)	112	122

The experiments with control macrophages taken as the reference = 100% ± S E M
Number of the experiments given in brackets
[a] cpm calculated per tissue nitrogen
[b] no macrophage preparation present

TABLE 4 *Effect of Macrophage Preparations on the Incorporation of Glucosamine into Granulation Tissue Slices or Cultured Granuloma Cells*

Experimental system and the addition	Incorporation into CPC precipitate per dry wt
Granulation tissue slices	
homogenized macrophages[a] (4)	129 (116–157)
3 day culture of granuloma cells	
medium of macrophages (3)	117 (110–125)
5 day culture of granuloma cells,	
medium of macrophages (3)	168 (142–190)

The average values with the ranges are given as percentage of the respective controls (= 100%) The granulation tissue slices were taken 7 days after implantation The number of experiments is given in brackets
[a] including one experiment with 7000 *g* sediment

supernatant of macrophage homogenate was fractionated with Sephadex G-100, the first-emerging non-dialysable fraction was inhibitory (data not shown)

For comparison, the granulation tissue slices were replaced by liver slices In liver also, the protein (including collagen) synthesis was decreased in the presence of homogenized macrophages (to about 85%), but was stimulated slightly by the pretreatment with SiO_2 (by 10–20% over the control with non-treated macrophages)

Effects of Various Materials on the Modulation of Protein Synthesis in Granulation Tissue Slices by Peritoneal Macrophages

Small-molecular substances Prostaglandins and oxygen atmosphere intensified the depressant effect of macrophages, whereas serotonin and cyclic AMP counteracted the effect (Table 2) The effect of serotonin and cyclic AMP cannot be due to direct action on the fibroblasts, since the medium was changed after pretreatment of the macrophages Bradykinin, actinomycin D, indomethacin (10^{-3}–10^{-5}M) did not influence the effect of macrophages

Macromolecular substances Many preparations have a SiO_2-like influence on the effect of macrophages on protein synthesis rheumatoid synovial fluid, connective tissue active peptide and extract from CCl_4-damaged liver (but not from normal ethanol-damaged or fatty liver) (Table 3) The effect of synovial fluid seems to be located in the first and last fractions in the gel filtration chromatography with Sephadex G-200

Experiments on the Effect of Macrophages on the Synthesis of Acid Mucopolysaccharides

Incorporation of glucosamine Table 4 shows the results of incorporation of glucosamine into the cetyl pyridinium chloride (CPC)-precipitated fraction, mainly hyaluronate, of granulation tissue slices during the proliferation phase (7 days) in the presence of macrophage preparations In more developed granulation tissue, the incorporation and also the enhancing effect of the macrophages were smaller The stimulating effect of the macrophages on the synthesis of acid mucopolysaccharides was not increased by pretreatment with SiO_2 One macrophage homogenate was fractionated to 500 *g*, *4000 g* and *7000 g sediments* and a *7000 g* supernatant The 7000 *g* sediment contained the stimulating agent, as did also the medium of cultured peritoneal macrophages (tested with cultured granuloma cells)

Experiments on the Effect of Macrophages on the Development of Granuloma in vivo

Table 5 shows that in the presence of *macrophage homogenate* the development of granuloma was retarded, as measured by the amounts of DNA RNA, nitrogen and uronic acid *The synthesis of* compounds containing hydroxyproline and hexosamine was increased In analogous experiments with *macrophage culture medium* in some cases there was again an increase of hydroxyproline, especially when calculated as the ratio of hydroxyproline to nitrogen Pretreatment of the macrophages with SiO_2 increased the *hydroxyproline/nitrogen ratio* further

The results of these experiments shown in Table 5 suggest that the macrophage material stimulates an increase of collagen in spite of a general decrease of nitrogen There might be an increase also in material containing hexosamine presumably in acid mucopolysaccharides

With *whole macrophages*, there was a different effect as regards the capacity for collagen synthesis (Table 6) The stimulus for the synthesis of collagen

TABLE 5 *Effects of Macrophage Preparations on Growth of Experimental Granulation Tissue for 3 Weeks*

Component analyzed µg/mg dry wt	Control		Macrophages Non treated		Macrophages SiO_2 treated	
A *Macrophage homogenate added*						
DNA	8 9 ± 0 34	(7)	8 7 ± 0 27	(12)	9 9 ± 0 35	(11)
RNA	1 8 ± 0 09	(8)	1 6 ± 0 04	(12)[a]	1 7 ± 0 06	(12)
Hydroxyproline	8 8 ± 0 38	(8)	10 8 ± 0 48	(12)[b]	9 9 ± 0 57	(12)
Nitrogen	63 7 ± 1 93	(8)	58 7 ± 2 83	(12)	47 8 ± 1 79	(12)[c]
Hydroxyproline/nitrogen	0 138		0 184		0 207	
Uronic acid	5 8 ± 0 21	(8)	5 3 ± 0 16	(12)	5 2 ± 0 22	(11)
Hexosamine	3 8 ± 0 18	(8)	4 7 ± 0 25	(12)[a]	4 3 ± 0 18	(12)
B *Medium of macrophage culture added*						
Hydroxyproline	8 2 ± 0 67	(8)	9 2 ± 0 52	(8)	9 03 ± 0 45	(8)
Nitrogen	86 8 ± 3 3	(8)	79 6 ± 2 9	(8)	72 5 ± 2 0	(8)[a]
Hydroxyproline/nitrogen	0 094 ± 0 007	(8)	0 117 ± 0 007	(8)	0 123 ± 0 008	(8)
Uronic acid	11 4 ± 0 23	(8)	11 1 ± 0 30	(8)	10 8 ± 0 28	(8)
Hexosamine	1 5 ± 0 02	(7)	1 5 ± 0 05	(8)	1 6 ± 0 05	(8)

The sponge implant was absorbed before the implantation with macrophage homogenate (A) or with the medium of cultured macrophages (B) possibly pretreated with SiO_2 particles as described in the text S E M and the number of experiments are indicated [a]$P < 0\ 05$ [b]$P < 0\ 01$, [c]$P < 0\ 001$ (with reference to control)

TABLE 6 *Incorporation of Labelled Proline by Slices from Granulation Tissue Grown into Sponge Implants Pretreated with Preparations from Untreated or SiO_2 treated Macrophages*

Compound analyzed	Macrophages Homogenized (3) Non treated	Macrophages Homogenized (3) SiO_2 treated	Macrophages Whole (2) Without SiO_2	Macrophages Whole (2) With SiO_2
Total protein per nitrogen	107 (100–112)	110 (90–141)	99 (99–99)	99 (86–112)
Hydroxyproline per DNA	62 (54–76)	41 (33–45)	122 (108–137)	159 (155–164)

The production of granulation tissue is described in the legend to Table 5 The data are given as percentage of the control The range and number of experiments are given in brackets

which was degraded in some way in the homogenized macrophages was apparently preserved in the whole macrophages, and even enhanced in those applied with SiO_2 (see Discussion) The macrophage homogenates may have contained activated collagenase (27)

DISCUSSION

Correlation with Previous Work

Anti inflammatory drugs have a variable effect on the release of lysosomal enzymes from macrophages at phagocytosis (8) Both platelets and phagocytic macrophages release into the cell culture medium factors which stimulate the proliferation of fibroblasts (20, 26) Artefacts due to the cold thymidine released from cells in dying culture and hence to dilution of the DNA [illegible] demons [illegible] prepara [illegible] variable in this study, although stimulation was always observed after SiO_2-pretreatment observed also by [illegible]

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There seems to be no information regarding the activity of RNase in the various states of macropha ges although considerable information has been accumulated concerning the effects of eliciting (17) Macrophages do not contain the RNase inhibitor destroyed by *p*-chloromercuribenzoate Whether the rheumatoid synovial fluid and other tissues contain

an inhibitor of macrophage RNase (7) remains to be studied

Macrophages release prostaglandins in response to inflammatory stimuli (8 14) In fact it is supposed that the »oxygen burst« at phagocytosis (6) contributes to the endoperoxide stages of prostaglandin metabolism It is interesting that exposure of macrophages to oxygen atmosphere increases their suppressing action on the fibroblasts Furthermore treatment of macrophages with prostaglandin (13) causes them to release cyclic AMP

Macrophages and the Fibrotic Process

The macrophages promote the synthesis of hyaluronate (Table 4) and DNA (19) potentiate the effects of connective tissue active peptide perhaps through the formation of prostaglandin and cyclic nucleotides (10 11) induce vascular proliferation when elicited (22) and enhance the chemotaxis at the »oxygen burst« (6) There is thus no doubt that macrophages prompt and stimulate the formation of granulation tissue (cf 3)

The exact role of macrophages in clinical fibroses such as those occurring in the synovial tissue or in liver remains to be studied Our present preliminary experiments show that the macrophage SiO_2 system stimulates the protein synthesis also in liver slices Liver contains more than half of the macrophages of the body in the form of the Kupffer cells but it is not yet defined whether their number or properties have any relationship to liver fibrosis The same can be said regarding the synovial fluid macrophages

The exact role of macrophages especially of their RNases in the human fibroses needs to be clarified The key point in our hypothesis that the acting mRNA of collagen and of other proteins is influenced directly or indirectly by RNases or other factors from macrophages has yet to be ascertained by direct measurement

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THE EFFECT OF HUMAN MILK FRACTIONS ON ROTAVIRUS IN RELATION TO THE SECRETORY IgA CONTENT

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Otnæss A B & Ørstavik I The effect of human milk fractions on rotavirus in relation to the secretory IgA content Acta path microbiol scand Sect. C 88 15-21 1980

Human milk from healthy Norwegian women was fractionated by ammonium sulphate precipitation and gel filtration The protein content lactoferrin and secretory IgA were measured Specific anti rotavirus IgA detected by indirect immunofluorescence was found in one out of five milk samples before fractionation while a more concentrated immunoglobulin fraction from the other four milk samples contained such IgA Before fractionation 3 of 5 milk samples neutralized human rota virus infection of LLC MK2 cells whereas concentrated IgA rich fractions of all 5 milk samples neutralized human rotavirus Some fractions without detectable IgA also neutralized human rotavirus This suggests that human milk contains rotavirus specific IgA as well as rotavirus neutralizing activity of non immunoglobulin nature

Key words Human milk secretory IgA rotavirus

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Breast fed infants suffer less from gastrointestinal disorders and upper respiratory tract infections than bottle fed infants (6) and human milk has been used to stop outbreaks of gastroenteritis caused by *E coli* O111 (17 18) Human milk has also been described as *containing specific antibodies against* certain viruses (13)

Human rotavirus was discovered in 1973 and has been established as one of the major causes of acute gastroenteritis in infants and children (8) *Chrystie et al* (2) found that rotavirus infections were significantly less frequent among breastfed babies than among bottle fed babies Rotavirus specific antibodies in human colostrum and milk have recently been *reported* (3 16 19 23) and *Thouless et al* (19) described a neutralizing effect of colostrum on human rotavirus in cell culture

Human milk may contain antiviral activity other than antibodies (11) to differentiate between such non antibody and specific antibody activity to rotavirus we have fractionated human milk and determined the secretory IgA (sIgA) content and the rotavirus neutralizing activity of the fractions

MATERIALS AND METHODS

Fractionation of milk (40-120 ml) was collected from 5 healthy mothers 5-40 days after delivery Cells and fat were removed by centrifugation (20000 × g/2h at 4 °C) and the supernatant fluid below the fat layer was carefully pipetted off Casein was precipitated at pH 4 6 and after centrifugation (13000 × g/30 min at 4 °C) trishydroxyaminomethane was added to the supernatant fluid to adjust the pH to 7 4 This step was *omitted* in milk samples Nos 2 3 and 4 without apparently influencing the results

An equal volume of saturated ammonium sulphate solution (4 °C) was added and the suspension was stirred for 15-18 h at 4 °C *After* centrifugation

RESULTS

Fractionation of human milk The milk samples Nos 1–4 were all obtained on days 5 and 6 after delivery, and the individual variation of the total protein sIgA and lactoferrin in the centrifuged milk (Fig 2) is similar to the observations of *McClelland et al* (12) Milk sample No 5 was obtained later in the lactation period (on day 40) and accordingly the milk had a lower content of protein, lactoferrin and sIgA than the other samples In the concentrated ammonium sulphate precipitate 25–50% of the total protein was sIgA, as compared to 5–16% sIgA in the centrifuged milk Most of the lactoferrin was retained in the ammonium sulphate supernatant which after concentration consisted of 43–57% lactoferrin

Three protein peaks were obtained after column chromatography of the immunoglobulin fraction (Fig 3a) Fused rocket immunoelectrophoresis showed that IgA was eluted in the first peak (Fig 3b) while lactoferrin and IgG were eluted in the second peak as indicated (Fig 3a) Peak I was usually not symmetrical and two separate pools

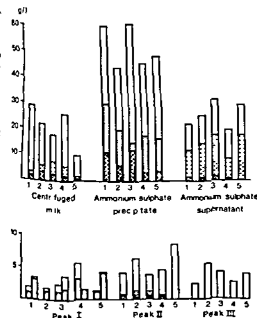


Fig 2 Total protein sIgA and lactoferrin in fractionated milk samples

Total protein ☐

sIgA ☐

lactoferrin ☐

were made accordingly No sIgA or lactoferrin were detected in peak III (Fig 2)

Anti-rotavirus IgA Rotavirus specific IgA, as determined by indirect immunofluorescence, was detected in only one of the five milk samples prior to fractionation (Table 1) In the ammonium sulphate precipitate, which is enriched in immunoglobulins, rotavirus specific IgA was detected in 4 out of 5 milk samples and after concentration of this fraction anti-rotavirus IgA was also detected in the fifth sample (Table 1) The titre of the rotavirus specific IgA varied independently of the variation of total sIgA in the concentrated ammonium sulphate fractions Sample No 5 had a titre of 16 and contained 16 5 g/l sIgA, whereas sample No 1 with a titre of 4 contained 30 g/l sIgA However, within the same milk sample, the specific anti rotavirus IgA titre varied in parallel with the total sIgA concentration

[illegible]

to dilution, since the sIgA concentration in the peak I was now about the same as in the centrifuged milk When peak I of milk 1 and milk 3 were further concentrated, rotavirus specific IgA was detected in both samples

Neutralization of rotavirus Three out of 5 samples of the centrifuged milk neutralized human rotavirus with a titre of 5 (Table 1) In the concentrated ammonium sulphate precipitate, all five samples contained neutralizing activity with titres of 10–40

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After ammonium sulphate precipitation, the concentrated supernatant also contained neutralizing activity in 4 of the 5 samples (titres 5–10) in spite of a lower concentration of total sIgA in the centrifuged milk, and the absence of detectable rotavirus specific IgA It should be noted that in milk No 5, the concentrated ammonium sulphate supernatant was without a detectable level of sIgA (0 01 [illegible]

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sIgA [illegible] in this peak.

DISCUSSION

Quantitative measurement of sIgA is often expressed in arbitrary units (15), or serum IgA is used as

(20000 × g/20 min at 4 °C) the supernatant was dialysed against phosphate buffer (0 01 M pH 7 4) (3 × 3 l) and the volume reduced about 20 times before testing The sediment was dissolved in a small volume of 0 01 M phosphate buffer, pH 7 4 and dialysed against the phosphate buffer After further concentration, aliquots of 1–2 ml of the immunoglobulin-rich fraction were chromatographed on an Ultrogel AcA 44 (LKB Sweden) column (70 × 2 cm) in phosphate buffered saline, pH 7 4 The protein peaks were pooled, concentrated and dialysed against phosphate buffer (0 01 M, pH 7 4)

Rocket immunoelectrophoresis was carried out on 9 × 23 cm glass plates with 1.5 mm gel thickness as described by *Weeke* (21) An electrophoresis apparatus with cooling (15 °C) was used The buffer was barbital/tris (pH 8 6) with ionic strenght 0 02 and the electrophoresis was run in 1% HSA agarose (Litex, Denmark) The gel contained 0 8% anti-human IgA specific for α-chain, made in swine (Orion, Finland) The samples (3 µl) were added while the voltage was kept at 2 v/cm The electrophoresis was continued for 16–48 h at 3 v/cm Experiments were performed in triplicate The plates were washed for 24 h in 0 1 M NaCl, and pressed and stained with Coomassie blue

Fused rocket immunoelectrophoresis was performed using 0 8% anti-IgA in the top gel, and with the holes (2 mm diameter) 1 cm from the top gels, 2 mm apart The samples were allowed to diffuse for 2 h at 20 °C, followed by electrophoresis as described above

Preparation of Anti-IgM Column and Anti-IgA Column

Sepharose 4 B (Pharmacia, Sweden) was activated by cyanogen bromide and anti IgM (32 mg in 3 ml) or anti-IgA (10 mg in 2 ml) (Dakopatts, Denmark) was coupled to 10 g activated gel by the method of *March et al* (10) The coupled gel was stirred with 1M ethanolamine and washed with 1) 0 1 M acetate buffer pH 4 0 2) 2M urea in 0 5 M NaCl 3) 0 1 M $NaHCO_3$ in 0 5 M NaCl, pH 9 5, 4) 0 2 M $NaHCO_3$, pH 9 5 and 5) 0 02 M phosphate buffer, pH 7 4 The capacity of the anti IgA affinity gel was *0 45 mg sIgA per ml packed gel*

Reference secretory IgA was prepared by further purification of the secretory IgA peak obtained after gel filtration of the milk sample The sIgA peak contained a small amount of IgM which was removed by passing the sample through the anti IgM affinity column or sIgA was retained on the anti IgA column and eluted with 1M citrate/phosphate buffer pH 2 8

The purified IgA was devoid of IgM IgG and lactoferrin and no impurities were detected by disc gel electrophoresis or crossed immunoelectrophoresis The sIgA preparation consisted of 77% 11S IgA, 5% 7S IgA and 18% larger polymer IgA determined by analytical ultracentrifugation

Secretory IgA was determined by rocket immunoelectrophoresis A linear standard curve was obtained in the range 0 1–4 g sIgA per l when 0 8% anti IgA (Orion) was used (Fig 1) When 0 16% antibody was used, 0 02 g secretory IgA/l (or 0 004 g serum IgA/l) could easily be measured (data not shown)

Reference serum IgA was obtained from Behringwerke, Germany

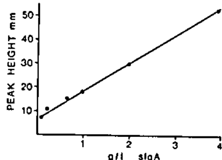


Fig 1 Standard curve for the determination of secretory IgA Rocket immunoelectrophoresis of 3 µl purified secretory IgA, diluted in 0 15 M NaCl The gel contained 0 8% anti human IgA prepared in swine The electrophoresis was run for 48 h at 3 v/cm

Rotavirus specific IgA was determined by an indirect immunofluorescent antibody technique using BSC-1 cells infected with a bovine strain of rotavirus as antigen (14) Milk fractions were either undiluted or diluted in phosphate buffered saline, and tested on virus infected and on noninfected cells The conjugate was FITC-labelled rabbit anti-human IgA (Dakopatts) diluted 1 20

Rotavirus neutralizing activity was measured in LLC-MK2 cells, as described by *Thouless et al* (20) with slight modifications Briefly, human rotavirus from the faeces of a child with acute gastroenteritis was diluted in Eagle's medium to about 100 infectious units per 0 025 ml and mixed with equal volumes of diluted milk fractions Following a 1 5 h incubation at 37 °C in 5% CO_2 atmosphere, 0 05 ml of the mixture was added in duplicate to flat-bottom microtiter plates (Lindbro 15 FP-96TC) with confluent monolayers of LLC-MK2 cells Eagle's medium (0 150 ml) containing 2% foetal calf serum, 0 80 mg $NaHCO_3$/ml and antibiotics were added, and the plates were sealed with adhesive tape and centrifuged (1100 × g/1 h) The plates were incubated *for 24 h at 37 °C and virus infected cells were stained by* indirect immunofluorescence technique using a gnotobiotic calf anti-rotavirus serum (H 48) kindly provided by Dr *G N Woode*, Iowa U S A

The number of virus infected cells was counted and the titre of the milk fractions read as the highest dilution which reduced the number of infected cells by 50% or more compared to the virus control without milk fraction

Lactoferrin was measured by single immunodiffusion where 1% HSA agarose gel contained 0 8% anti lactoferrin (Dakopatts) or by rocket immunoelectrophoresis as described above using 0 8% anti lactoferrin in the gel The antigen holes were 2 mm in diameter

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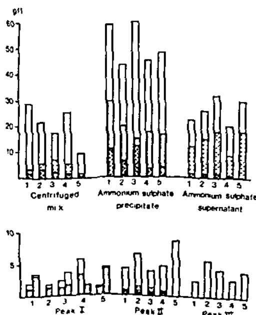


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When an aliquot of the concentrated sediment of milk No 5 was passed through an anti-IgA affinity column and concentrated to the original volume of the aliquot, no rotavirus IgA was detected and the neutralizing titre was reduced from 40 to 5

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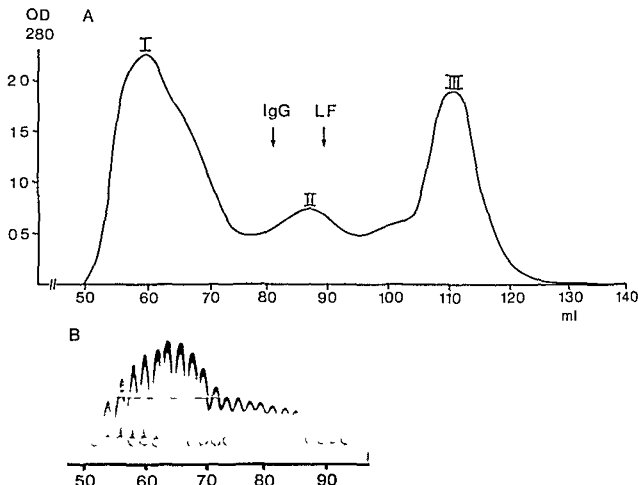


Fig 3 Centrifuged milk was chromatographed on an AcA Ultrogel column A Elution of protein B Elution of secretory IgA measured by fused rocket electrophoresis

reference IgA (4) giving too low values, unless correction factors are used (1,7) Purified secretory IgA is well suited as reference for the measurement of sIgA, and no conversion factors are needed

The fractionation of the milk made it possible to study the effect on rotavirus of (1) centrifuged milk, (2) an immunoglobulin-rich fraction, (3) a lactoferrin-enriched fraction, (4) isolated sIgA (Peak I), and (5) an immunoglobulin- and lactoferrin-depleted fraction (Peak III)

No rotavirus specific IgA was detected by immunofluorescence in 3 of the 5 centrifuged milk samples (Table 1), which is in agreement with the results of *Thouless et al* (19) who observed that on day 5–6 after delivery the specific IgA content was very low or absent Similarly, *Schoub et al* (16) found rotavirus specific IgA in only 10% of the milk samples 2 days after delivery This apparent lack of specific antibodies is probably due to low sensitivity of the test system rather than to the absence of specific antibody, as in the present study the concentrated immunoglobulin fraction of all the milk samples contained rotavirus specific IgA When more sensitive assays are used, such as radioimmunoassay (3) or enzyme linked immunosorbent assay (23) rotavirus antibodies have been detected in the majority of unconcentrated milk samples

For the neutralization test we used human rotavirus obtained from one patient, probably representing a single strain of the virus However, there are at least two serotypes of human rotavirus (22) which both give rise to antibody production early in life Since such infections are extremely common, it is most likely that the milk samples contain antibodies against both serotypes

The rotavirus neutralization activity was highest in the concentrated immunoglobulin-rich fractions After removal of IgA in one of these fractions, the neutralizing titre was highly reduced, suggesting that IgA was of major importance for the neutralizing activity of this milk Neutralizing

TABLE 1 *Specific sIgA and Neutralizing activity of Human Milk against Rotavirus*

	MILK 1			MILK 2			MILK 3			MILK 4			MILK 5		
	Total sIgA g/l	Specific sIgA[a]	Rotavirus neutralization[b]	Total sIgA g/l	Specific sIgA[a]	Rotavirus neutralization[b]	Total sIgA g/l	Specific sIgA[a]	Rotavirus neutralization[b]	Total sIgA g/l	Specific sIgA[a]	Rotavirus neutralization[b]	Total sIgA g/l	Specific sIgA[a]	Rotavirus neutralization[b]
Centrifuged Milk	3.2	0	5	3.4	4	5	1.9	0	< 5	2.5	0	5	0.5	0	< 5
Ammonium sulphate precipitate	3.2	1	20	4.8	4	5	1.7	1	< 5	2.0	0	5	1.2	2	10
Concentrated ammonium sulphate precipitate	30.0	4	20	20.0	8	10	17.5	≤[c]	10	15.0	4	10	16.5	16	40
Concentrated ammonium sulphate supernatant	1.6	0	10	1.6	4	< 5	0.6	0	5	1.5	0	5	0	0	5
Peak I a	1.1	0	< 5	1.3	≤1[c]	< 5	3.2	0	5	1.3	0	5	1.2	4	5
Peak I b	3.2	0	5	1.3	≤1[c]	< 5	1.5	0	5	1.7	1	5	4.2	8	10
Peak II	0.7	0	< 5	0.8	0	< 5	0.6	0	5	0.6	0	< 5	0	0	< 5
Peak III	0	0	< 5	0	0	< 5	0	0	5	0	0	5	0	0	< 5

Milk samples 1–4 were obtained 5–6 days after delivery, and milk sample 5 40 days after delivery
Peak I–III are pooled fractions after column chromatography

[a] Rotavirus specific IgA is expressed as the highest dilution with positive immunofluoresence staining

[b] Neutralization of rotavirus is expressed as the highest dilution which reduced the number of infected cells by 50 %

[c] Immunofluoresence reading not possible due to unspecific staining of noninfected cells

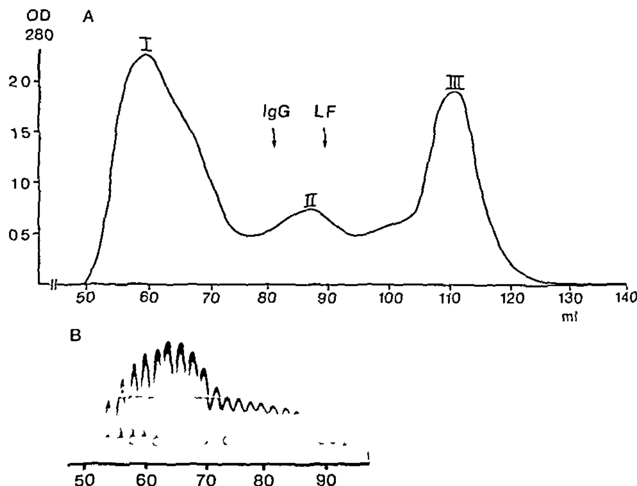


Fig 3 *Centrifuged milk was chromatographed on an AcA Ultrogel column A Elution of protein B Elution of* secretory IgA measured by fused rocket electrophoresis

reference IgA (4) giving too low values, unless correction factors are used (1,7) Purified secretory IgA is well suited as reference for the measurement *of sIgA, and no conversion factors are needed*

The fractionation of the milk made it possible to study the effect on rotavirus of (1) centrifuged milk, (2) an immunoglobulin-rich fraction (3) a lactoferrin-enriched fraction, (4) isolated sIgA (Peak I), and (5) an immunoglobulin- and lactoferrin-depleted fraction (Peak III)

No rotavirus specific IgA was detected by immunofluorescence in 3 of the 5 centrifuged milk samples (Table 1), which is in agreement with the results of *Thouless et al* (19) who observed that on day 5–6 after delivery the specific IgA content was very low or absent Similarly, *Schoub et al* (16) found rotavirus specific IgA in only 10% of the milk samples 2 days after delivery This apparent lack of specific antibodies is probably due to low sensitivity of the test system rather than to the absence of specific antibody as in the present study *the concentrated immunoglobulin fraction of all the* milk samples contained rotavirus specific IgA When more sensitive assays are used, such as *radioimmunoassay (3) or enzyme linked immuno*sorbent assay (23) rotavirus antibodies have been detected in the majority of unconcentrated milk samples

For the neutralization test we used human rotavirus obtained from one patient, probably representing a single strain of the virus However, there are at least two serotypes of human rotavirus (22) which both give rise to antibody production early in life Since such infections are extremely common, it is most likely that the milk samples contain antibodies against both serotypes

The rotavirus neutralization activity was highest in the concentrated immunoglobulin rich fractions After removal of IgA in one of these fractions, the neutralizing titre was highly reduced, suggesting that IgA was of major importance for the neutralizing activity of this milk Neutralizing

TABLE I *Specific sIgA and Neutrali ing activity of Human Milk against Rotavirus*

	MILK I			MILK 2			MILK 3			MILK 4			MILK 5		
	Total sIgA g/l	Specific sIgA[a]	Rotavirus neutral ization[b]	Total sIgA g/l	Specific sIgA[a]	Rotavirus neutral ization[b]	Total sIgA g/l	Specific sIgA[a]	Rotavirus neutral ization[b]	Total sIgA g/l	Specific sIgA[a]	Rotavirus neutral ization[b]	Total sIgA g/l	Specific sIgA[a]	Rotavirus neutral ization[b]
Centrifuged Milk	3 2	0	5	3 4	4	5	1 9	0	< 5	2 5	0	5	0 5	0	< 5
Ammonium sulphate precipitate	3 2	1	20	4 8	4	5	1 7	1	< 5	2 0	0	5	1 2	2	10
Concentrated ammonium sulphate precipitate	30 0	4	20	20 0	8	10	17 5	≤[c]	10	15 0	4	10	16 5	16	40
Concentrated ammonium sulphate supernatant	1 6	0	10	1 6	4	< 5	0 6	0	5	1 5	0	5	0	0	5
Peak I a	1 1	0	< 5	1 3	≤1[c]	< 5	3 2	0	5	1 3	0	5	1 2	4	5
Peak I b	3 2	0	5	1 3	≤1[c]	< 5	1 5	0	5	1 7	1	5	4 2	8	10
Peak II	0 7	0	< 5	0 8	0	< 5	0 6	0	5	0 6	0	< 5	0	0	< 5
Peak III	0	0	< 5	0	0	< 5	0	0	5	0	0	5	0	0	< 5

Milk samples 1–4 were obtained 5–6 days after delivery and milk sample 5 40 days after delivery
Peak I–III are pooled fractions after column chromatography

[a] Rotavirus specific IgA is expressed as the highest dilution with positive immunofluoresence staining
[b] Neutralization of rotavirus is expressed as the highest dilution which reduced the number of infected cells by 50%
[c] Immunofluoresence reading not possible due to unspecific staining of noninfected cells

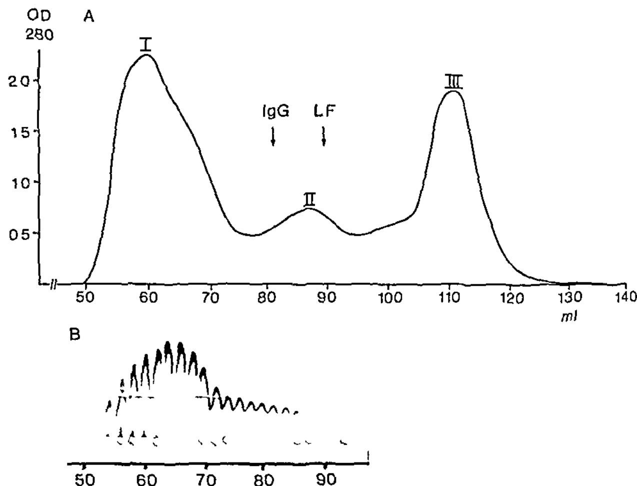


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activity was also found in fractions with low or undetectable sIgA (Table 1) indicating that other *factor(s) besides immunoglobulins* may be of importance in the virus neutralization The concentrated supernatants are rich in lactoferrin, which accounts for 40–60% of the total protein Lactoferrin, alone or in combination with other factors, may be of some importance On the other hand, lactoferrin is usually thought to act as a bacteriostatic agent via depletion of iron This hypothesis cannot easily explain an effect on rotavirus, and the actual agent may be another compound in these fractions

Some milk samples had a rotavirus neutralizing activity in fractions after gel filtration where no sIgA was detected It is unlikely that the neutralizing activity of Peak *III* (elution volume corresponding to mol wt 40 000) was due to low molecular weight (monomer) IgA, since monomer IgA would be eluted in Peak II rather than in Peak III It is likely that Peak III contains a non immunoglobulin anti-virus activity and this represents further evidence for the importance of other factors in the promotion by human milk of resistance to viral infections The activity may be analogous to that reported in milk towards other viruses (11) The nature of the non-immunological neutralizing activity is under current investigation

This paper reports studies on milk fractions from which fat and cells have been removed An antivirus activity of lipid nature has been reported (5) and it is possible that significant resistance factors may also be found in the lipid and cellular fractions

In conclusion we have demonstrated that human milk contains anti rotavirus activity of IgA as well as of non immunoglobulin nature

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Analytical ultracentrifugation was kindly performed by dr *T B Christensen* Biochemical Institute University of Oslo Oslo Norway

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Acta path microbiol scand Sect. C 88 23–29 1980

THE *IN VITRO* RESPONSE OF LYMPHOCYTES FROM ADENOID VEGETATIONS AND TONSILS TO PPD INFLUENCE OF AUTOLOGOUS BLOOD MONOCYTES, T LYMPHOCYTES AND UNSEPARATED LYMPHOCYTES

HANS HENRIK MOGENSEN KAREN INGER MEISTRUP LARSEN UGGI MEISTRUP LARSEN and VAGN ANDERSEN

Department of Infectious Diseases M and Medical Department TA Rigshospitalet, Copenhagen and the Departments of Otolaryngology Hvidovre Hospital and Sundby Hospital Denmark

Mogensen H H Meistrup-Larsen K I Meistrup-Larsen U & Andersen V The *in vitro* responses of lymphocytes from adenoid vegetations and tonsils to PPD Influence of autologous blood monocytes T lymphocytes and unseparated lymphocytes Acta path microbiol scand Sect. C 88 23–29 1980

Thymidine incorporation in lymphocytes obtained from adenoids (AVL) tonsils (TL) and blood (*PBL*) and stimulated by PPD was compared in 19 BCG vaccinated and 17 unvaccinated patients Responses were approximately tenfold higher in vaccinated than in unvaccinated patients The responses obtained for AVL/TL were lower than for PBL in BCG vaccinated as well as in unvaccinated patients In autologous mixtures of AVL/TL and PBL a linear increase in thymidine incorporation was obtained with increasing numbers of PBL. The monocyte concentration in cell suspensions obtained from adenoid and tonsil tissue was lower than in cells prepared from blood addition of autologous monocytes purified from blood to cultures of AVL and TL was followed by *a small increase in* response to PPD but not to the level of responsiveness of PBL Addition of autologous T lymphocytes purified from blood to cultures of AVL and TL was followed by a small increase in thymidine incorporation of similar proportions in cultures with and without PPD It is concluded that the proportion of PPD sensitized lymphocytes is smaller in AVL/TL *than in PBL*.

Key words Adenoid vegetations tonsils lymphocyte response PPD *in vitro*

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Received 18 vii 79 Accepted 21 ix 79

In man *in vitro* studies of cell mediated immunity to microorganisms have mostly been performed employing blood lymphocytes Recently increasing attention has been paid to the examination of cells obtained from lymphoid organs but as yet little information is available concerning the specific reactivity of such cells to defined antigens

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MATERIALS AND METHODS

Subjects 30 patients admitted for adenoidectomy and 5 patients ad[illegible] the patien[illegible] vaccinate[illegible]

Acta path microbiol scand Sect. C 88 23–29 1980

THE *IN VITRO* RESPONSE OF LYMPHOCYTES FROM ADENOID VEGETATIONS AND TONSILS TO PPD INFLUENCE OF AUTOLOGOUS BLOOD MONOCYTES, T LYMPHOCYTES AND UNSEPARATED LYMPHOCYTES

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MATERIALS AND METHODS

Subjects 30 patients admitted for adenoidectomy and 6 patients admitted for tonsillectomy were studied Among the patients undergoing adenoidectomy 13 were BCG vaccinated (average age 9 years) and 17 were unvaccina

ted (average age 4 years) The patients undergoing tonsillectomy were older (average age 14 years) and were all BCG-vaccinated This vaccination was carried out at about 7 years of age The patients were admitted to the Departments of Otolaryngology at Hvidovre Hospital and at Sundby Hospital for adenoidectomy (because of recurrent upper respiratory infections, enlargement of the adenoid or a combination of both) or for tonsillectomy (because of recurrent tonsillitis) The persons were in good health at the time of the investigation, in particular without any signs of infection None received any drug treatment Skin testing with tuberculin was not performed

Lymphocyte cultures Blood and tissue were obtained at operation as previously described (9) The adenoid or tonsil tissue removed was placed in RPMI 1640 medium with penicillin, streptomycin and heparin Within 1 hour a cell suspension was prepared by gently pressing the tissue through a steel mesh Mononuclear cells were isolated on a Ficoll-Isopaque gradient

The lymphocyte transformation technique has been reported (8) Most of the lymphocyte preparations employed in this study have been described earlier (9) including quantitation of T and B lymphocytes (7), according to the results of cell titrations and kinetic experiments in the previous study (9), 10^5 cells were cultured in a volume of 500 µl for 5 days when stimulated with PPD, whereas cultures with polyclonal activators were terminated after 3 days ^{14}C-thymidine incorporation was quantitated by liquid scintillation counting, the results given are means of triplicate determinations, after subtraction of the values obtained in unstimulated cultures unless otherwise indicated A response in a stimulated culture is considered positive when it is more than 2 5 times higher than in the corresponding unstimulated culture

15% normal human serum, were distributed to plastic Petri dishes After incubation in a humidified atmosphere containing 5% CO_2 for 90 minutes at 37 ° non adherent cells were removed by flooding the plate 5 times with medium, prewarmed to 37 °C Thereafter adherent cells were resuspended by gentle scraping with a rubber policeman

Monocytes were identified by α naphthyl esterase staining (5) of cytocentrifuged smears The proportion of esterase positive cells was on the average 71% the yield in the monocyte purification step was 67% and the viability as determined by trypan blue exclusion was 91%

Separation and identification of T lymphocytes For purification of T lymphocytes the method described by *Greaves & Brown* (1) was employed Fenwal Leukopak nylon fibers were rinsed for 4 days in distilled water at 37 °C, the water was changed once daily After drying, 2 g of fiber were employed per plastic syringe of 10 ml RPMI 1640 containing 15% human serum was added to the fiber-filled syringes, the mononuclear cell suspension, containing $10-50 \times 10^6$ cells in a volume of 5 ml was added drop wise, and the column was incubated for 30 minutes at 37 °C Thirty ml of prewarmed medium were used for slow elution of cells

T lymphocytes were identified by rosetting with sheep erythrocytes The proportion of E-rosetting cells was on the average 99%, the viability 98%

Microbial antigen PPD (RT 33 and RT 23) was a gift from *Mogens Magnusson* Statens Seruminstitut Copenhagen In all experiments, a dose titration was carried out 8 concentrations of PPD were used, ranging from 5 µg to 2 3 ng per culture with 3-fold dilution steps The thymidine incorporation given is the maximal response obtained by any of the varying doses unless otherwise indicated

Polyclonal activators The polyclonal activators were employed in the optimal concentrations (9) 50 µl of the 500 ml reconstituted stock solution of phytohaemagglutinin (PHA-P, Difco), 100 µg of pokeweed mitogen (PWM, Grand Island Biological Co) and 20 µg of Concanavalin-A (Con-A, Pharmacia) A dose titration was carried out with PHA

Statistical methods For comparison of lymphocyte responses, the Wilcoxon rank sum test was employed In testing for correlations the Kendall rank sum test was employed

RESULTS

Unstimulated Cultures

Thymidine incorporation in unstimulated cultures of AVL was compared in BCG-vaccinated and unvaccinated patients No differences were found at 3 or 5 days of culture ($p > 0\ 05$) (Table 1) Likewise there was no difference between unstimulated PBL from vaccinated and unvaccinated patients

Comparison of PPD-stimulated Cultures of AVL/TL and PBL

Cultures were stimulated by PPD employing a dose titration in each experiment In the vaccinated patients a positive response was obtained with AVL/TL in 81%, and with PBL in 100% among the unvaccinated responses were either absent or low (Table 2) The differences in response between vaccinated and unvaccinated are highly significant ($p < 0\ 001$) for AVL/TL as well as for PBL In the vaccinated group no correlation was observed between the responses to PPD and the age of the donor

It appears from Table 2 that the responses obtained for AVL were lower than for PBL both in vaccinated and in unvaccinated children ($p < 0\ 001$) For the vaccinated patients, the concentration of

TABLE 1 *Thymidine Incorporation by Lymphocytes from 10 BCG vaccinated and 17 Unvaccinated Patients Polyclonal Activators were Employed in the Optimal Concentration PHA Further in 1 Supraoptimal and 2 Suboptimal Concentrations*

	AVL c p m (±SD)		PBL c p m (±SD)	
	BCG vaccinated	unvaccinated	BCG vaccinated	unvaccinated
PHA 2	10490 (±2791)	11806 (±3581)	22811 (±6035)	21442 (±7653)
1	12649 (±2738)	14050 (±4003)	24444 (±4342)	23875 (±8922)
1 4	4722 (±3249)	3253 (±2651)	8534 (±5040)	5342 (±3515)
1 8	690 (±967)	425 (±646)	1362 (±1224)	1097 (±1479)
PWM	2250 (±723)	1519 (±835)	4145 (±1656)	2961 (±1564)
Con A	8022 (±1793)	7347 (±2999)	14981 (±6756)	14526 (±6600)
unstimulated 3 days	19 (±17)	33 (±24)	59 (±35)	17 (±24)
5 days	47 (±51)	48 (±36)	262 (±222)	132 (±102)

PPD inducing maximal thymidine incorporation was the same in AVL and in PBL (Fig 1) in the unvaccinated patients a higher PPD concentration (5 μg/500 μl) was needed to induce maximal thymidine incorporation No correlation was found between PPD induced thymidine incorporation in PBL and AVL in the vaccinated children

In the smaller group of patients all BCG vaccinated who underwent tonsillectomy the responses were similarly lower in TL than in PBL ($p < 0.01$) The TL responses were compared to the AVL responses in the vaccinated patients and no difference was found ($p > 0.05$)

Mixed Cultures of Blood and Tissue Lymphocytes

AVL (2 cases) and TL (3 cases) obtained from BCG-vaccinated patients were mixed with autologous PBL and stimulated with 0 5 μg PPD per culture Nineteen different proportions of the two cell types were used always employing 10^5 cells per

TABLE 2 *Responses to PPD of Lymphocytes Obtained from Adenoid Vegetations (AVL) Tonsils (TL) and Blood (PBL) The Patients are Divided According to their BCG vaccination Status*

Cell type	Status of BCG vaccination	No of patients with a positive response/no tested	Maximal thymidine incorporation c p m mean	range
AVL		5/17	25	(0–61)
PBL		9/17	672	(0–3055)
AVL	+	8/10	268	(25–670)
PBL		10/10	5345	(252–19082)
TL	+	5/6	322	(79–1176)
PBL		6/6	4132	(2959–5557)

ted (average age 4 years) The patients undergoing tonsillectomy were older (average age 14 years) and were all BCG-vaccinated This vaccination was carried out at about 7 years of age The patients were admitted to the Departments of Otolaryngology at Hvidovre Hospital and at Sundby Hospital for adenoidectomy (because of recurrent upper respiratory infections, enlargement of the adenoid or a combination of both) or for tonsillectomy (because of recurrent tonsillitis) The persons were in good health at the time of the investigation, in particular without any signs of infection None received any drug treatment Skin testing with tuberculin was not performed

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Separation and identification of monocytes Mononuclear cells, isolated from the blood and suspended at a concentration of 4×10^6/ml in RPMI 1640 containing 15% normal human serum were distributed to plastic Petri dishes After incubation in a humidified atmosphere containing 5% CO_2 for 90 minutes at 37 ° non-adherent cells were removed by flooding the plate 5 times with medium prewarmed to 37 °C Thereafter, adherent cells were resuspended by gentle scraping with a rubber policeman

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TABLE 3 *Influence of Addition of Autologous Monocytes Purified from the Blood on Thymidine Incorporation by AVL/TL Stimulated with 0 5 µg PPD*

Person	Age (years)	Response of PBL c p m		Response of AVL/TL c p m	Addition of monocytes[a] Response of AVL/TL after addition of the following proportion of plastic adherent cells 5% c p m	10% c p m	15% c p m	% esterase positive cells in suspension added
AP	12	4210[b]	AVL	37	332	270	332	68
AR	11	716	AVL	217	360	202	188	60
SF	9	1588	TL	34	65	116	138	80
AG	15	4380	TL	44	499	424	362	44
MS	14	3661	TL	168	1019	571	585	69
BJ	8	2642	AVL	266 (58)	422 (102)	888 (72)	922 (157)	79
MA	27	3796	TL	859 (110)	1667 (1531)	3074 (1278)	2989 (2322)	78
AS	10	5103	TL	604 (152)	1202 (201)	1160 (301)	1070 (228)	81
TC	10	2664	TL	481 (89)	740 (135)	935 (175)	626 (154)	79
	mean ± SD	3196 ± 1413		301 ± 289	701 ± 508	849 ± 909	801 ± 879	71 ± 12

[a] In 4 of the patients monocytes were added to identical cultures without PPD (thymidine incorporation given in parentheses)

[b] The values given are not corrected for thymidine incorporation in unstimulated cultures which for PBL was mean 157 c p m (range 38–365 c p m) and for AVL/TL 72 c p m (range 37–152 c p m)

TABLE 4 *Influence of Addition of Autologous T Lymphocytes Purified from the Blood on Thymidine Incorporation by AVL/TL the Cultures were Stimulated by 0 5 µg PPD The Values Given are not corrected for Thymidine Incorporation in the Corresponding Unstimulated Cultures (Given in Parentheses)*

Person	Response of PBL c p m	Response of AVL/TL c p m	Response of AVL/TL after the addition of 5% T lymphocytes c p m	10% T lymphocytes c p m	15% T lymphocytes c p m
BJ	2642 (136)	266 (58)	512 (105)	491 (93)	235 (91)
MA	3796 (172)	859 (110)	1600 (503)	1954 (258)	2175 (475)
AS	5103 (191)	604 (152)	1209 (251)	1577 (352)	1741 (273)
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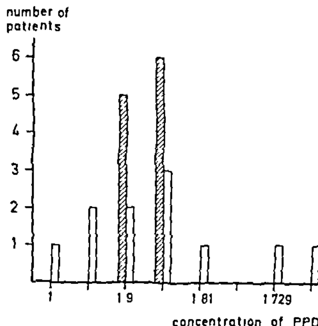


Fig 1 Optimal concentrations of PPD for stimulation of lymphocytes obtained from adenoids / tonsils (open columns) and from blood (hatched columns) in 11 BCG vaccinated patients undergoing adenoidectomy or tonsillectomy Concentration 1 is equal to 5 μg of PPD per 500 μl

culture In all 5 cases a linear increase in thymidine incorporation was obtained with increasing content of PBL A typical result is shown in Fig 2

Influence of Monocytes

The proportion of esterase positive cells was considerably smaller in the tissue cell suspension than in blood mononuclear cells In 9 patients the content of esterase positive cells was in mean 13% (range 6–25%) in PBL and 3% (range 1–5%) in AVL/TL To investigate whether the low responsiveness to PPD of AVL/TL as compared to PBL was related to this difference plastic adherent cells were purified from blood and added to cultures of AVL or TL from the same individual (Table 3) Addition of 5% plastic adherent cells was followed by a moderate but statistically significant increase in thymidine incorporation ($p<0\ 001$) A further increase of plastic adherent cells to 10% and 15% (in some cases even to 30%) was not followed by any further increase in thymidine incorporation In 4 of the patients (BJ MA AS and TC) monocytes were added to identical cultures without PPD In these patients this addition did not increase thymidine incorporation in unstimulated cultures except for the patient MA (the only adult)

In cultures of purified monocytes (10^5 per 500 μl) no response to 0 5 μg PPD was found in 3 BCG vaccinated persons Mean thymidine incorporation 149 c p m (cultures without PPD 150 c p m)

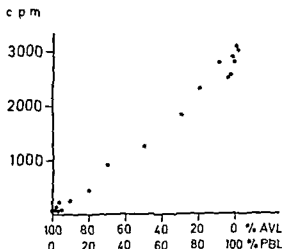


Fig 2 Thymidine incorporation in mixtures of autologous lymphocytes obtained from adenoids (AVL) and blood (PBL) of a BCG vaccinated patient Each culture contained 10^5 cells and was stimulated by 0 5 μg PPD

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T lymphocytes were purified from the blood of 4 BCG vaccinated patients by passing the mononuclear cells through a nylon fiber column and added in various concentrations to autologous cultures of AVL or TL (Table 4) In PPD stimulated cultures addition of 5% T lymphocytes was followed by a doubling of thymidine incorporation A similar increase was seen in identical cultures without PPD Addition of increasing numbers of T lymphocytes (10% and 15%) was not followed by any further increase in thymidine incorporation

In cultures of purified T lymphocytes (10^5 per 500 μl) high responses to 0 5 μg PPD were found in 3 BCG vaccinated persons Mean thymidine incorporation 6129 c p m (cultures without PPD 722 c p m)

Polyclonal Activators

Thymidine incorporation in cultures of AVL and PBL stimulated by PHA PWM and Con A was compared in BCG vaccinated and unvaccinated patients (Table 1) No differences were found except for a slightly lower response to PWM in the unvaccinated group

The dose response pattern for PHA was similar in cultures of AVL and PBL

DISCUSSION

It has previously been demonstrated that the response of blood lymphocytes to purified protein derivative of tuberculin is well correlated to the

TABLE 3 *Influence of Addition of Autologous Monocytes Purified from the Blood on Thymidine Incorporation by AVL/TL Stimulated with 0 5 µg PPD*

Person	Age (years)	Response of PBL c p m		Response of AVL/TL c p m	Addition of monocytes[a]			
					Response of AVL/TL after addition of the following proportion of plastic adherent cells			% esterase -positive cells in suspension added
					5% c p m	10% c p m	15% c p m	
AP	12	4210[b]	AVL	37	332	270	332	68
AR	11	716	AVL	217	360	202	188	60
SF	9	1588	TL	*34*	*65*	*116*	*138*	*80*
AG	15	4380	TL	44	499	424	362	44
MS	14	3661	TL	168	1019	571	585	69
BJ	8	2642	AVL	266 (58)	422 (102)	888 (72)	922 (157)	79
MA	27	3796	TL	859 (110)	1667 (1531)	3074 (1278)	2989 (2322)	78
AS	10	5103	TL	604 (152)	1202 (201)	1160 (301)	1070 (228)	81
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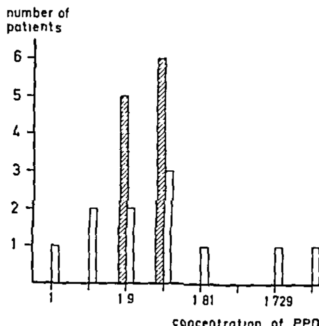


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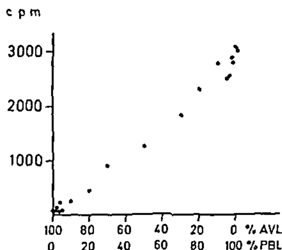


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degree of tuberculin sensitivity in the cell donor (4) This antigen was employed in the present study for stimulation of lymphocytes isolated from adenoids tonsils and blood in patients with known status of BCG vaccination Much higher responses were found in vaccinated than in unvaccinated patients this was demonstrated for PBL as well as for AVL/TL (Table 2) The major part of PPD responsiveness is specific In tuberculin skin test negative individuals blood lymphocyte responses to PPD were maximally 10% of the responses in persons with a positive skin test (3) The data presented here demonstrate that also AVL/TL are specifically stimulated in the vaccinated patients

No important differences were found between BCG vaccinated and unvaccinated patients as regards lymphocyte responses after stimulation with polyclonal activators or in unstimulated cultures

Responses of AVL and TL to PPD were much lower than those of PBL This was found in vaccinated as well as in unvaccinated patients Similar findings were made by *Sugiyama et al* (11) Contrary to this *Mazuran el al* (6) found higher responses to PPD in TL and to a lesser degree in AVL than in PBL The patients examined in the latter study were all BCG vaccinated immediately after birth and the experimental conditions were different The cell density and the concentration of PPD were markedly higher cultures were incubated for 96 hours and the transformation response was evaluated as a percentage of blast like cells in this latter study

In mixed cultures of AVL/TL and autologous PBL it was demonstrated that thymidine incorporation following PPD stimulation was directly correlated to the number of PBL in the mixtures This suggests that the low responsiveness to PPD of AVL/TL is due to lower numbers of responding cells rather than the effect of differences in suppressor or helper cells

As reported previously (7) the concentration of monocytes was much lower in cell suspensions obtained from adenoids and tonsils than from blood adherent cells purified from blood and added in various numbers to autologous cultures of AVL or TL did not raise the response to the level of PBL responsiveness Yet a small but significant increase was seen after a 5% increase of the monocyte concentration in AVL or TL So the monocyte macrophage activity in the tissue cell suspension seems insufficient for optimal lymphocyte stimulation in this *in vitro* system but the differences between responses to PPD of AVL/TL and of PBL cannot be explained quantitatively this way These results are in agreement with the findings of *Sugiyama et al* (11) who reduced the concentration of adherent cells (on absorbent cotton) in preparations of lymphocytes isolated from tonsils and blood of persons with a positive skin test to tuberculin Responses to PPD were slightly reduced in PBL and markedly reduced or absent in TL

The proportion of T lymphocytes in cell suspensions obtained from adenoids and tonsils has been found slightly lower than in the blood (2 6 7 10) Further qualitative differences could be present T lymphocytes purified from blood were therefore added to cultures of AVL and TL Only a small increase in thymidine incorporation was seen which was of similar proportion in PPD stimulated and unstimulated cultures

In conclusion AVL and TL demonstrated a considerably smaller proliferative response to PPD than did PBL Addition of autologous monocytes or T lymphocytes purified from the blood caused only small increases in the responses of AVL/TL Thus the discrepancy between tissue and blood cells seems due not to a deficiency of helper cells but to a smaller proportion of PPD sensitized lymphocytes in the tissue cell suspension

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HUMAN T CELL RESPONSE TO HERPES SIMPLEX VIRUS ANTIGEN *IN VITRO*

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Berle, E. J. Jr. & Thorsby, E. Human T cell response to herpes simplex virus antigen *in vitro*. Acta path. microbiol. scand. Sect. C 88: 31–37, 1980.

Herpes simplex virus type I antigen (HSV Ag) added to T cells from individuals with clinical history of recurrent herpes labialis causes a proliferative response *in vitro*. This T cell response requires presensitization of the responding cell donor and will occur only in the presence of adherent cells (macrophages). The intensity of the response is closely related to the number of adherent cells present, being optimal at a ratio of 10:1 between T cells and adherent cells. Preliminary studies also indicate that the response to HVS Ag is restricted by the HLA-D/DR determinants of the T cell donor.

Key words: T cell response; herpes simplex virus antigen; human.

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Previous investigations by our group have dealt with different aspects of T lymphocyte responses towards antigens (*Bergholtz & Thorsby* 1977, 1978, 1979) and haptens (*Thorsby & Nousiainen* 1979) *in vitro*. Using purified protein derivative (PPD) of tuberculin, it was shown that this antigen caused a proliferative response of T lymphocytes from sensitized individuals provided that adherent cells (macrophages) were present. More important, the T-macrophage cooperation required that the macrophages expressed at least one of the HLA-D/DR molecules of the T cell donor. The HLA-D restriction of this response was recently confirmed by *Hansen et al.* (1978).

Since HLA-D/DR restriction of the antigen-specific proliferative response of T cells from sensitized individuals has as yet only been demonstrated for PPD, it was necessary to confirm these observations using another antigen. We selected herpes simplex virus (HSV) type I antigen because previous studies had shown that T cell enriched populations from patients with recurrent herpes labialis will proliferate in response to HSV Ag in vitro (*Kirchner et al.* 1978, *Shillitoe et al.* 1978). The degree of adherent cell dependency of this response, however, had not been studied in detail.

Studies of T cell immune response to HSV in vitro are also important because little is known of the role of different immune responses in protection against recurrent HSV infection. For instance, [illegible] *upp* 1974).

The aim of the studies reported here was to (1) investigate whether the T cell proliferative response to HVS antigen *in vitro* was dependent on adherent cells, (2) whether individuals with and without clinical history of recurrent herpes labialis differed in their T cell responsiveness, and (3) whether the response was restricted by the HLA-D/DR determinants of the T cell donor.

MATERIALS AND METHODS

Cell Donors

Blood samples were taken from otherwise healthy members of the hospital staff. Cell donors were grouped according to the presence or absence of anamnestic evidence of recurrent herpes labialis. State of sensitization was confirmed by the presence of complement binding antibodies with titre >8 or neutralizing antibody titre >4.4. The donors without anamnestic

evidence of previous infection all had complement binding antibodies with titre <8 or neutralizing antibodies with titre <2 Complement binding reaction was performed by the standard technique and with commercially available antigen (Orion Diagnostica Helsinki Finland) The neutralizing test was performed using 100 tissue culture infectious doses of HSV hominis type I on vero line of African green monkey kidney cells Serology testing was performed by Dr *M Degré* Institute of Bacteriology The National Hospital Oslo

Herpes Simplex Virus Antigen

We have used lyophilized herpes simplex type I CF (complement fixation) antigen produced in monkey kidney cells (Orion Diagnostica Helsinki Batch D I Cat no D 428) The control antigen used (Orion Diagnostica Batch D I Cat no D 711) has been processed corresponding to the CF antigen but it does not contain herpes simplex type I virus antigen

HLA D and HLA DR Typing

Typing of the cell donors was performed with techniques and reagents previously described (*Albrechtsen et al* 1978)

Cell Separation Techniques

Defibrinated blood was separated by the Ficoll Isopaque flotation technique (*Boyum* 1968) using Lymphoprep (Nyco Oslo Norway) The peripheral blood mononuclear cells (PBM) thus obtained were washed twice at 600 × G in RPMI 1640 (Gibco Biocult Glasgow Scotland)

Latex Ingestion

One hundred µl latex (Bacto Latex 0 81 Difco Laboratories Michigan USA) was added to 10 ml RPMI 1640 Cell suspensions were adjusted to 1×10^6 per ml Equal parts of cell and latex suspension were incubated for 30 min at 37 °C and the number of latex ingesting cells per 100 was counted

T Cell Preparation

The PBM were suspended in tissue culture medium i e RPMI 1640 with 100 I U penicillin/ml 100 µg/ml streptomycin and 20% normal serum from a pool of healthy blood donors This serum was not screened for anti herpes virus antibodies The cells were then incubated in 25 cm^2 flat bottomed tissue culture flasks (Cat no 3013 Falcon Cal USA) for 2 h at 37 °C Following this incubation the non adherent cells were pipetted off and made to form rosettes with AET treated sheep red blood cells (SRBC) (*Pellegrino et al* 1976) The adherent cells were incubated further (see later) The rosettes were gently resuspended in RPMI 1640 without serum and separated from non-T cells by Ficoll Isopaque flotation The SRBC were lysed with normal serum containing anti sheep xeno antibodies washed twice and resuspended in tissue culture medium This cell suspension was incubated in flat bottomed tissue culture flasks overnight at 37 °C in order to remove further remaining adherent cells After incubation for approx 20 h the non adherent cells were pipetted off and washed twice in RPMI 1640 at 600 × G The adherent cells from this preparation were discarded In order to obtain pure T cell populations the non adherent cells were again made to form rosettes with AET treated SRBC After lysing the SRBC with normal serum the cells were washed twice resuspended in tissue culture medium and adjusted to 1×10^6 cells per ml This cell population was designated »T cells More than 80% of these formed rosettes with AET treated SRBC less than 2 % ingested latex and less than 1% were Ig positive 95% of the cells proved viable by means of Trypan blue exclusion

Preparation of Adherent Cells

The adherent cells were obtained from the PBM after the initial incubation for 2 h and from the non T fraction following the first step of the T cell preparation After overnight incubation at 37 °C 3 3 µl/ml EDTA was added and the cells were loosened by vigorous pipetting after incubation on ice for 90 min The cells were washed three times at 4 °C and 500 × G resuspended in tissue culture medium adjusted to 1×10^5 cells per ml and kept on ice until used Of these adherent cells designated »Macrophages« (M ø) more than 85% ingested latex To inhibit any contributing proliferation of the adherent cells these were always irradiated with 2000 rad before use

Cell Culture Techniques

Flat bottomed microtitre plates were used (TC disc 3590 Costar USA) in all experiments A constant number of 5×10^4 T cells was added to each well and adherent cells (M ø) were added in a decreasing number from 50 000–500 per well HVS Ag antigen was diluted in tissue culture medium and decreasing concentrations of antigen were prepared Twenty µl of these solutions was added to each well making a final volume of 170 µl and final HSV Ag concentrations ranging from 1 10 to 1 10^6 To test the T cell responses to nonspecific mitogens cultures were incubated for 3 days with Phytohaemagglutinin (PHA) (Wellcome Research Lab England) in final concentrations of 1 100 per well

All combinations were performed in triplicate and cultured from 2–15 days at 37 °C After the set number of days in culture 0 2 ml 1 0 µCi of ^3H thymidine was added to each well and after an additional 24 h of culture the plates were harvested with a semi automatic multiple harvester (Skatron Lierbyen Norway) Incorporation of ^3H thymidine was assessed by liquid scintillation counting and the results were expressed as counts per minute (cpm) ± S E or as median cpm (to save space)

RESULTS

T Cell Stimulation by HSV Ag

In order to study the T cell proliferative capacity to HSV-Ag and to ascertain whether or not the response is dependent on adherent cells 5×10^4 T

TABLE 1 *Proliferative Response of T Cells to HSV-Ag in vitro of Cell Donors with Clinical History of Recurrent Herpes Labialis*

T cell donor	Anti HSV[1] antibodies	HSV-Ag[2]	T cells alone[3]	T + MØ cells[4]
EB	27	–	72 ± 18[5]	363 ± 26
		+	281 ± 38	3,174 ± 65
UH	13	–	95 ± 30	1 212 ± 309
		+	661 ± 37	31 859 ± 572
ABT	16	–	428 ± 9	235 ± 94
		+	506 ± 196	4 185 ± 347
ET	19	–	325 ± 160	268 ± 31
		+	1 493 ± 634	15 836 ± 882
NW	4 4	–	207 ± 20	134 ± 1
		+	181 ± 59	11 997 ± 138

[5] incorporation of 3H thymidine after culture for 6 days expressed as cpm ± S E of triplicates

cells from nine donors with clinical history of recurrent herpes labialis were tested All donors had an increased level of anti-herpes virus antibodies (see »Materials and Methods«) The results of 6-day cultures obtained using cells from five of the donors are shown in Table 1 In all five cell donors, a strong T cell response to HVS Ag was seen provided that autologous adherent cells were also present. Antigen added to T cells alone caused no or only a weak response (donors UH and ET) There was no response to the control antigen Similar results were obtained using T cells and adherent cells from an additional four donors with clinical history of ... fr ... Ag (data not shown)

The T Cell Response Requires Presensitization of the T Cell Donor

Having found a T cell response to HSV-Ag in sensitized donors we tested nine individuals without clinical history of recurrent herpes labialis as well as four neonates The results of representative experiments are shown in Tables 2 and 3 Neither cell donors without clinical history of recurrent herpes labialis (Table 2) nor neonates (Table 3) were found to respond to HSV Ag, regardless of whether autologous adherent cells were present or not As a control, the proliferative capacity of T cells from these non responding donors was tested using PHA, this was found to be with ... nec ... sor ... ha ... column of Table 3)

Dose-response Investigations

The proliferative response of the T cells was linearly correlated to the final concentration of HSV-Ag The antigen did not appear to be toxic, even at final concentrations of up to 1 10 Based on these data an antigen concentration of 1 100 was used in subsequent experiments.

In order to study further the macrophage dependency of the anti HSV response, we investigated the influence of the number of macrophages on the T cell response, using a constant number (50 000) of responding T cells and adherent cells in

evidence of previous infection all had complement binding antibodies with titre <8 or neutralizing antibodies with titre <2 Complement binding reaction was performed by the standard technique and with commercially available antigen (Orion Diagnostica Helsinki Finland) The neutralizing test was performed using 100 tissue culture infectious doses of HSV hominis type I on vero line of African green monkey kidney cells Serology testing was performed by Dr *M Degré* Institute of Bacteriology The National Hospital Oslo

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One hundred µl latex (Bacto Latex 0 81 Difco Laboratories Michigan USA) was added to 10 ml RPMI 1640 Cell suspensions were adjusted to 1 × 10^6 per ml Equal parts of cell and latex suspension were incubated for 30 min at 37 °C and the number of latex ingesting cells per 100 was counted

T Cell Preparation

The PBM were suspended in tissue culture medium i e RPMI 1640 with 100 I U penicillin/ml 100 µg/ml streptomycin and 20% normal serum from a pool of healthy blood donors This serum was not screened for anti herpes virus antibodies The cells were then incubated in 25 cm^2 flat bottomed tissue culture flasks (Cat no 3013 Falcon Cal USA) for 2 h at 37 °C Following this incubation the non adherent cells were pipetted off and made to form rosettes with AET treated sheep red blood cells (SRBC) (*Pellegrino et al* 1976) The adherent cells were incubated further (see later) The rosettes were gently resuspended in RPMI 1640 without serum and separated from ›non-T cells by Ficoll Isopaque flotation The SRBC were lysed with normal serum containing anti sheep xeno antibodies washed twice and resuspended in tissue culture medium This cell suspension was incubated in flat bottomed tissue culture flasks overnight at 37 °C in order to remove further remaining adherent cells After incubation for approx 20 h the non adherent cells were pipetted off and washed twice in RPMI 1640 at 600 × G The adherent cells from this preparation were discarded In order to obtain pure T cell populations the non adherent cells were again made to form rosettes with AET treated SRBC After lysing the SRBC with normal serum the cells were washed twice resuspended in tissue culture medium and adjusted to 1 × 10^6 *cells per ml This cell* population was designated »T cells More than 80% of these formed rosettes with AET treated SRBC less than 2 % ingested latex and less than 1% were Ig positive 95% of the cells proved viable by means of Trypan blue exclusion

Preparation of Adherent Cells

The adherent cells were obtained from the PBM after the initial incubation for 2 h and from the non T fraction following the first step of the T cell preparation After overnight incubation at 37 °C 3 3 µl/ml EDTA was added and the cells were loosened by vigorous pipetting after incubation on ice for 90 min The cells were washed three times at 4 °C and 500 × G resuspended in tissue culture medium adjusted to 1 × 10^5 cells per ml and kept on ice until used Of these adherent cells designated »Macrophages‹ (M ø) more than 85% ingested latex To inhibit any contributing proliferation of the adherent cells these were always irradiated with 2000 rad before use

Cell Culture Techniques

Flat bottomed microtitre plates were used (TC disc 3590 Costar USA) in all experiments A constant number of 5 × 10^4 T cells was added to each well and adherent cells (M ø) were added in a decreasing number from 50 000–500 per well HVS Ag antigen was diluted in tissue culture medium and decreasing concentrations of antigen were prepared Twenty µl of these solutions was added to each well making a final volume of 170 µl and final HSV Ag concentrations ranging from 1 10 to 1 10^6 To test the T cell responses to nonspecific mitogens cultures were incubated for 3 days with Phytohaemagglutinin (PHA) (Wellcome Research Lab England) in final concentrations of 1 100 per well

All combinations were performed in triplicate and cultured from 2–15 days at 37 °C After the set number of days in culture 0 2 ml 1 0 µCi of ^{3}H thymidine was added to each well and after an additional 24 h of culture the plates were harvested with a semi automatic multiple harvester (Skatron Lierbyen Norway) Incorporation of ^{3}H thymidine was assessed by liquid scintillation counting and the results were expressed as counts per minute (cpm) ± S E or as median cpm (to save space)

RESULTS

T Cell Stimulation by HSV-Ag

In order to study the T cell proliferative capacity to HSV Ag and to ascertain whether or not the response is dependent on adherent cells 5 × 10^4 T

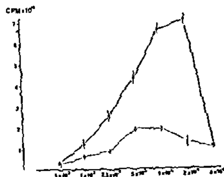


Fig 1 Influence of number of macrophages on the T cell response to HSV-Ag 5×10^4 T-enriched cells were co cultured with varying quantities of autologous macrophages (shown on the abscissa) and stimulated with HSV Ag (final concentration 1 100) All cultures were harvested on day 6 and the results are expressed as mean cpm ± s e

Kinetics

Fig 2 shows the results of four experiments involving two cell donors with, and two without a clinical history of recurrent herpes labialis The strongest response was seen in the two cell donors with clinical history of recurrent herpes labialis The peak of the proliferative response was between day 6 and day 10 A slight response was seen in one of the two negative controls

The T Cell Response Appears to be HLA-D Restricted

Our results show that the T cell response to HSV-Ag requires sensitization of the T cell donor and cooperation from adherent cells These observations provided an opportunity to study whether the HSV-Ag specific T cell response was restricted by the self HLA-D/DR determinants of the T cell donor T cells and macrophages from unrelated HLA-D/DR typed donors were mixed Table 4 shows the data from two typical experiments indicating that HSV-Ag together with allogeneic but HLA D/DR identical or compatible macrophages will induce an antigen-specific response similar to that seen in autologous mixtures In contrast, in fully HLA D/DR disparate combinations, no or only slight HSV-Ag response was seen There was some response in combinations where the T cell and macrophage donor shared one of their two HLA-D/DR determinants, but this was usually lower than in allogeneic HLA-D/DR identical combinations Similar results were obtained using other cell donors Details of these studies will be published elsewhere

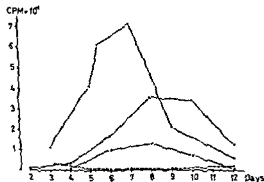


Fig 2 Kinetics of the macrophage-dependent T cell response to HSV Ag 5×10^4 T-enriched cells were co-cultured with 5×10^3 autologous macrophages and stimulated with HSV Ag (final concentration 1 100) for

HSV antibodies

●———● T + Mø from cell donor with history of possible one attack of herpes labialis and with anti HSV antibodies

●- - - - -● T + Mø from cell donor without history of herpes labialis and without anti HSV antibodies

All results expressed as median cpm of triplicates (to save

DISCUSSION

Our experiments show that T cells from sensitized individuals will respond to HSV type I Ag in vitro The results reported by *Shillitoe et al* (1978) and *Kirchner et al* (1978) also suggested that the proliferative lymphocyte responses to HSV-I *in vitro* were mediated by T cells Furthermore, our studies demonstrate that the HSV-Ag T cell response is dependent on adherent cells, since T cells alone do not proliferate when stimulated with HSV Ag Macrophages alone did not proliferate when confronted ...

... has not been tested in our study

As can be seen from Table 1, there is a great deal of variation in the intensity of the T cell response to HSV-Ag This may be due to differences in the immunological status of the cell donors, since the stage of disease was not taken into account. Sometimes a slight HSV-Ag specific response was also seen in the T cell population alone (donors UH and ET, Table 1) Having already noted (Fig 2) that as little as 2% Mø are able to mediate a T cell response to HSV-Ag it seems reasonable to assume

TABLE 2 *Proliferative Response of T cells to HSV Ag in vitro of Cell Donors without Evidence of Previous Herp Labialis*

T cell donor	Anti HSV antibodies	HSV Ag	T cells alone	T + Mø cells
AB	2	–	194 ± 86	488 ± 115
		+	496 ± 235	249 ± 86
KG	2	–	208 ± 74	194 ± 46
		+	293 ± 147	396 ± 233
OJB	2	–	1 200 ± 351	2 076 ± 169
		+	1 535 ± 617	2 154 ± 522
TM	not tested	–	101 ± 14	71 ± 5
		+	299 ± 53	165 ± 9
MS	<2	–	174 ± 34	224 ± 30
		+	205 ± 33	362 ± 21

Legends and culture conditions as given in Table 1

decreasing quantities from 50 000–500 Fig 1 shows the results of two of a series of four experiments giving similar results Even at a T/Mø ratio of approximately 50 1 some HSV Ag response of T cells was seen The optimal T cell response occurred at T Mø ration between 10 1 and 10 5 decreasing at higher concentrations of macrophages

TABLE 3 *Proliferative T Cell Response to HSV Ag in Neonates*

T cell donor	HSV Ag	T cells alone	PBM or T + Mø cells [1)]	PHA response[2)]
Neonate A	–	86 ± 16	1 835 ± 118	5 627 ± 754
	+	69 ± 14	2 269 ± 313	3 049 ± 812
Neonate B	–	335 ± 134	1 916 ± 334	23 529 ± 3 109
	+	756 ± 130	2 232 ± 68	24 219 ± 2 218
Neonate C	–	165 ± 41	279 ± 52	109 451 ± 5 019
	+	301 ± 61	267 ± 91	60 478 ± 3 801
Neonate D	–	141 ± 19	102 ± 22	62 406 ± 3 111
	+	131 ± 31	169 ± 42	48 580 ± 1 501

1) In neonates A & B 5 10^4 PBM were used
In neonates C & D 5 10^4 T cells + 5×10^3 macrophages were used
2) Same number of cells as described under 1)

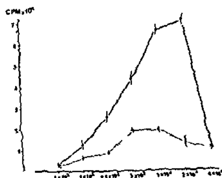


Fig 1 Influence of number of macrophages on the T cell response to HSV Ag 5 × 10⁴ T-enriched cells were co cultured with varying quantities of autologous macro phages (shown on the abscissa) and stimulated with HSV Ag (final concentration 1 100) All cultures were harvested on day 6 and the results are expressed as mean cpm ± s e

Kinetics

Fig 2 shows the results of four experiments involving two cell donors with, and two without a clinical history of recurrent herpes labialis The strongest response was seen in the two cell donors with clinical history of recurrent herpes labialis The peak of the proliferative response was between day 6 and day 10 A slight response was seen in one of the two negative controls

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Our results show that the T cell response to HSV-Ag requires sensitization of the T cell donor and cooperation from adherent cells These observa tions provided an opportunity to study whether the HSV-Ag specific T cell response was restricted by the self HLA-D/DR determinants of the T cell donor T cells and macrophages from unrelated HLA-D/DR typed donors were mixed Table 4 shows the data from two typical experiments indicating that HSV-Ag together with allogeneic but HLA-D/DR identical or compatible macrophages will induce an antigen-specific response similar to that seen in autologous mixtures In contrast in fully HLA D/DR disparate combinations no or only slight HSV-Ag response was seen There was some response in combinations where the T cell and macrophage donor shared one of their two HLA-D/DR determinants, but this was usually lower than in allogeneic HLA-D/DR identical combina tions Similar results were obtained using other cell donors Details of these studies will be published elsewhere

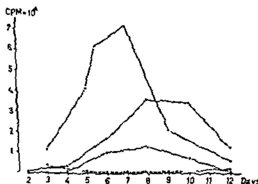


Fig 2 Kinetics of the macrophage-dependent T cell response to HSV Ag 5 × 10⁴ T-enriched cells were co cultured with 5 × 10³ autologous macrophages and stimulated with HSV Ag (final concentration 1 100) for

●———● T + M ø from cell donor with history of possible one attack of herpes labialis and with anti HSV antibodies

●- - - - -● T + M ø from cell donor without history of herpes labialis and without anti HSV antibodies

All results expressed as median cpm of triplicates (to save space)

DISCUSSION

Our experiments show that T cells from sensitized individuals will respond to HSV type I Ag in vitro The results reported by *Shillitoe et al* (1978) and *Kirchner et al* (1978) also suggested that the proliferative lymphocyte responses to HSV I *in vitro* were mediated by T cells Furthermore, our studies demonstrate that the HSV-Ag T cell response is dependent on adherent cells *since T cells* alone do not proliferate when stimulated with HSV-Ag Macrophages alone did not proliferate when confronted with HSV-Ag Similarly, *Shillitoe et al* (1978) found that nylon wool purified T cells responded less well to HVS *in vitro* Whether B cells will proliferate when confronted with HSV-Ag *in vitro* has not been tested in our study

As can be seen from T

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HS

immunological status of the cell donors since the stage of disease *was not* taken into account Sometimes a slight HSV-Ag *specific* response

to HSV-Ag, it seems reasonable *to assume*

TABLE 4 *HLA-D Restriction of T Cell Response to HSV-Ag*

HSV-Ag[1]	T cells[2] alone	T cells + MØ[3] autologous	T cells + MØ allogeneic				
		DR 1/7	DR 1/7	DR 1/1	DR 1/3	DR 2/7	DR 3/3
–	177 ± 49	180 ± 31	803 ± 185	98 ± 14	2,393 ± 657	9,993 ± 2744	1,356 ± 31
+	889 ± 378	17,417 ± 3230	13,935 ± 2700	17,092 ± 918	9,426 ± 876	15,185 ± 1170	3 051 ± 930
		17,237[4]	13,132	16,994	7,033	5,193	1 695
		DR 1/8	DR 1/8		DR 3/8		DR 5/6
–	299 ± 56	343 ± 42	200 ± 26		3,818 ± 426		4 080 ± 1 589
+	294 ± 46	28,194 ± 3877	25,652 ± 4,963		12,600 ± 387		5,093 ± 970
		27,851	25,452		8,782		114

1) Final Ag concentration 1 100
2) 5×10^4 T cells
3) 5×10^3 adherent cells
4) Incremental cpm

that this phenomenon is due to slight macrophage contamination of the T cell population

Lopez & O'Reilly (1977) showed that individuals with recurrent disease and healthy individuals with neutralizing antibodies to HSV 1 responsed to antigen in culture Normal individuals without neutralizing antibodies responsed with a significantly lower response We found no significant T cell response in individuals without history of herpes labialis Furthermore, T cells from newborns (Table 3) did not respond to HSV-Ag, even in the presence of adherent cells The response to PHA, however, was found to be within normal limits Our HSV-Ag was not mitogenic for T cells *Kirchner et al* (1978) also found no lymphoproliferative response to HSV-Ag using cord blood However, a response was obtained using *C parvum*

It is interesting to note the higher »background« (i e without antigen) in cells from neonates A and B than from neotates C and D In the former two experiments PBM were used, and the higher »back ground« compared to T cells plus MØ in the latter experiments may be due to spontaneous prolifera tion of B lymphocytes

Our experiments indicate further that the adhe rent cell-dependent T cell response to HSV Ag is restricted by self-HLA-D determinants We were able to show in other studies that the lack of cooperation between cells from HLA-D/DR disparate donors was not due to suppression or cytotoxicity generated as the result of HLA-D/DR disparity between the T cells and adherent cells Details of these studies will be published elsewhere These results correspond well with the findings previously reported for the macrophage-dependent PPD response of sensitized T cells (*Bergholtz & Thorsby* 1977 and 1978, *Hansen et al* 1978)

Studies in mice have shown that the cytotoxic T cell response to virus infected cells is restricted by products of the H-2 complex (*Zinkernagel* 1978) Recent studies also indicate that at least two T cell sub-sets determine the resistance to HSV in mice (*Howes et al* 1979) Studies are needed to see whether the same is true in man The experimental setup reported here appears to be well suited for these purposes, particularly since the same antigen may by used for studies of the HLA-D restriction of the proliferative response and the possible HLA-ABC restriction of cytotoxic T cells towards HSV-Ag expressing target cells

This work was supported by grants from the *Norwegian Society for Fighting Cancer* and the *Norwegian Council for Science and the Humanities* We are grateful for the technical assistance of *Helena Nousiainen*

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Acta path microbiol scand Sect C 88 39–45 1980

EXPERIMENTAL *ESCHERICHIA COLI* 06 INFECTION IN MICE

I Effect of Immunisation on Resistance in Relation to 06 Antibody Levels in Mice of Different Strains

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Ahlstedt S Experimental *Escherichia coli* 06 infection in mice I Effect of immunisation on resistance in relation to 06 antibody levels in mice of different strains Acta path microbiol scand Sect. C 88 39–45 1980

The susceptibility to intraperitoneal infection with *E coli* 06 k2 bacteria and the increase of resistance after immunization and immune serum injection was analysed in eight mouse strains (CBA A/J C3H C3H/HeJ C57 Bl/6J congenitally athymic C57 Bl nu/nu and their heterozygous nu/+ litter mates as well as NMRI mice) A different susceptibility to the infection was found among the strains This was not related to endotoxin resistance or thymus deficiency or to the ability of the animals to form antibodies as measured with the enzyme linked immunosorbent assay ELISA Immunization of the animals with 5×10^7 *E coli* 06 K13 bacteria resulted in a smaller increase in resistance in the less susceptible CBA mice than in the more susceptible A/J or C3H/HeJ mice This pattern was further accentuated after repeated immunization The development of resistance by immunization seemed independent of T-cells since the nu/nu mice were as resistant as the nu/+ mice The nu/nu mice however formed less antibodies after vaccination than did their nu/+ litter mates The lowest antibody responses were noted in the NMRI mice but this was accompanied with similar increase in resistance compared with the other strains forming 10 fold more 06 antibodies Immunization with as little as 10^2–10^4 bacteria also resulted in a rise in resistance This was however accompanied by a minute increase in antibody titer Despite content of minute antibody levels administration of such immune serum gave protection of the recipients It was concluded that very small amounts of antibodies would provide protection against *E coli* infection varying from one mouse strain to another

Key words Experimental infection *Escherichia coli* 06 antibody level mice

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Received 20 viii 79 Accepted 1 x 79

In systemic infection with *Escherichia coli* bacteria it is well known that various defence factors are of importance These factors of resistance can provide efficient killing and elimination of bacteria as well as increase the resistance against the endotoxin (1 2 4 7 11–14 16–18 21 22) The mechanisms involved in the defence against bacterial infections have not been settled despite extensive investigations (11) but genetic control has been indicated (8–10 18–20) Activation of the immune system in the host by the bacteria has been shown to result in protection both through the formation of antibodies (1, 3 12 13) and by stimulation of cellular factors (1, 3 4 14 19) Antibodies presumably of high avidity against the somatic 0 antigen (1 3) or K antigen (12) are efficiently protective In different individuals however, the antibody levels have not been found closely related to the level of protection regardless of what antibody assay used (for review see 16)

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In the present investigation the 06 antibody

amounts and avidities estimated with a very sensitive enzyme linked immunosorbent assay *ELISA (5) were related* to the resistance of various mouse strains to intraperitoneal E coli infection after immunization or administration of immune serum in a system leaving the K antibodies without effect A protective effect of minute amounts of antibodies was indicated to be differently efficient in different mouse strains but also other obviously cell bound factors seemed to contribute

MATERIAL AND METHODS

Animals Inbred SPF mice 6–8 weeks of age of both sexes of the strains CBA (Anticimex Stockholm Sweden) C3H/TIF C3H/HeJ C57Bl/6J congenitally athymic C57Bl/6J nu/nu and heterozygous litter mates nu/+ (Fries Bomholt Gard Lichtenberg Denmark) as well as outbred NMRI mice (Anticimex) were used

Antigens Bacteria of the *E coli* 06 K13 H1 strain WHO designation Su 4344/41 or *E coli* 015 K? bacteria (not K13 or K2a2c) the latter strain originally isolated from a patient were used for immunization and for serologically unspecific stimulation respectively The bacteria were killed using 0 5 per cent formalin for 24 h and then washed before use For infection experiments *E coli* 06 K2a 2c H1 bacteria WHO designation Su 1242 were used

Treatment of animals Groups of 50 or 60 mice were used untreated as controls or were injected once or twice with 30 day intervals with 5 × 10^7 *E coli* bacteria killed in formalin Four days after the last injection with killed bacteria the mice were infected (40 or 50 animals/group) or bled through the retroorbital plexus or by cervival dislocation without infection (10 animals/groups) Immune serum was similarly prepared by immunizing groups of 100–150 animals which were bled 4 days after treatment These sera were administered intraperitoneally i p or in some experiments intravenously i v in 0 4 ml aliquots The serum was given undiluted or at 1/4 1/8 or 1/16 dilutions to recipients which were infected (50 animals 10 per bacterial dose see below) or bled (10 animals) the day after serum transfer

Infection assay E coli 06 K2a 2c H1 bacteria were grown overnight in Tryptose Phosphate Broth and then

amounts of free endotoxin Forty or in most experiments 50 mice were divided into five groups in which each mouse was infected i p with 0 5 ml of the bacteria serially diluted 4 fold in broth The highest bacterial dose given was about 5 × 10^8 bacteria The mortality was recorded after 48 hours since no additional deaths occurred later on The results of untreated CBA mice were used as reference The results were expressed as the dilution of bacteria giving LD_{50} permitting statistical analysis of the results with Spearman Karber s test using a computer (PDP 8 E Digital Equipment Co Maynard Mass) (1) This infection assay shows a good reproducibility and the results for untreated controls of a particular strain were 30 ± 4 ($p < 0.05$) as based on 8 experiments The corresponding results for immunized animals were 58 ± 6 ($p < 0.05$)

In this assay only live bacteria cause any deaths of the animals and no effects of released substances from dead bacteria can be seen The infected animals have bacteremia as ascertained by blood samples cultured on blood agar plates (unpublished) In this model utilizing *E coli* 06K13 for immunization and 06K2a2c for infection only the 0 antibodies are protective (13)

Antibody assay The enzyme linked immunosorbent assay (5) was employed as previously described (2) using boiled antigen from *E coli* 06 K2a 2c H1 or Re antigen extracted as described by *Galanos et al* (6) The antibody titers were recorded as the highest serum dilution reciprocal giving 0 25 enzymatic activity units at 405 nm The relative average antibody avidities were recorded using the same assay by measuring the time in minutes required for 50 percent of the antibodies to bind (2)

RESULTS

Sensitivity of Different Strains of Mice to Infection with E coli *Bacteria in Relation to Antibody Levels and Antibody Avidities after Immunization*

The susceptibility to the *E coli* 06 K2a 2bH1 bacteria was determined for the seven strains of mice employed The A/J and C3H/HeJ mice were significantly more susceptible than all the other strains the C3H/TIF and C57Bl/6J were slightly less susceptible while the CBA nu/nu nu/+ and NMRI were more resistans This pattern did not relate to the ability of the animals to respond by antibody formation against the 06 antigen (Table I) Thus the susceptible A/J mice formed antibodies of similar amounts and avidities as the relatively resistant CBA mice while the susceptible C3H/HeJ formed antibodies of the highest avidities among the strains tested (Table I)

Effect of Immunization on the Resistance against Infection in the Various Strains of Mice

One injection of *E coli* 06 bacteria induced antibody formation and also protection in all animals A significant increase of the resistance in the immunized mice was noted Immunization of the A/J and C3H/HeJ mice resulted in a more accentuated increase of the resistance than in the other strains The rise in antibody levels was however similar in the mouse strains tested except for the nu/nu mice and in particular the NMRI mice in which a smaller antibody response was noted The avidities of the 06 antibodies formed by the various strains were also similar except for the C3H/HeJ animals where higher avidities were

TABLE 1 *The Dilution of Bacterial Suspension for LD_{50} in Mice of Various Inbred Strains to E coli 06 Infection before and after Immunization The 06 Antibody Titers and Avidities (within Brackets) are Given n d Represents not Done The Avidities are Given as the Time in Minutes for 50% of the Antibodies to Bind*

	Untreated animals		Animals immunized at							
			day 0				day 0 and 30			
					antibody levels after 4 days				antibody levels after 34 days	
Mouse strain	Group	Dilution of bacterial suspension for LD_{50}	Group	Dilution of bacterial suspension for LD_{50}	IgG[a)]	IgM[b)]	Group	Dilution of bacterial suspension for LD_{50}	IgG[c)]	IgM[b)]
CBA	1	1/50	9	1/5[f)]	10 (16)	2 000 (12)	15	1/2[h)]	1000 (1)	10 000 (1)
A/J	2	1/140[d)]	10	1/15[f)g)]	10 (18)	1 000 (12)	16	1/5[h)]	80 n d	900 (1)
C3H	3	1/90[e)]		n d	<10	700 (13)		n d	n d	n d
C3H/HeJ	4	1/140[d)]	11	1/5[f)]	<10	1 000 (4)	17	1/1[h)]	400	5 000
C57Bl	5	1/90[e)]		n d	<10 (13)	600		n d	n d	n d
nu/nu	6	1/45	12	1/10[f)]	<10	300 (10)		n d	n d	n d
nu/+	7	1/40	13	1/5[f)]	<10	1 000 (10)		n d	n d	n d
NMRI	8	1/50	14	1/10[f)]	<10	92 (12)		n d	n d	n d

a) IgG antibodies measured with Fc specific anti mouse IgG
b) IgM antibodies measured with μ specific anti mouse IgM
c) IgG antibodies measured with anti mouse IgG (heavy and light chain)
d) Significantly different from groups 1 3 5–8
e) Significantly different from [illegible]
f) [illegible]
g) [illegible]
h) [illegible]

TABLE 2 *Dilution of Bacterial Suspension for LD_{50} and Titers of 06 Antibodies in Various Mouse Strains after Transfer of Immune Serum n d Represents not Done*

Donor strain	Recipient strain	Antibody titer in recipient after transfer of serum				Dilution of bacterial suspension for LD_{50} after transfer of immune serum	
		undiluted		diluted 1/8		undiluted	diluted 1/8
		IgM	IgG	IgM	IgG		
CBA	CBA	390	58	n d	n d	1/5[a)c)]	n d[b)]
C3H/HeJ	C3H/HeJ	675	380	34	<10[a)c)]	1/15[c)]	
A/J	A/J	70	50	n d	n d	1/10[a)c)]	n d
CBA	C3H/HeJ	215	75	n d	n d	1/15[a)c)]	n d
C3H/HeJ	CBA	625	440	41	10	1/10[a)c)]	1/5[a)c)]
CBA	A/J	210	130	n d	n d	1/5[a)c)]	n d

a) [illegible]
b) [illegible]
c) [illegible] same strain

noted (Table 1) Antibodies of the IgM but not the IgG class *against the Re structure* could be found but only in very small amounts after the immunization (results not shown)

Induction of an anamnestic immune response in the CBA, A/J and C3H/HeJ mice resulted in a further increase of resistance against infection in all strains tested This was most accentuated for the C3H/HeJ mice (Table 1) The raised antibody amounts and avidities were not found to be closely related to the increase of resistance (Table 1) Thus a slightly smaller increase in antibody titers was found for the C3H/HeJ mice compared to the CBA mice (Table 1) No antibodies to the Re structure could be recorded even after boosting with a 30 day interval between the injections (results not shown)

Effect of Transferred Antibodies on the Resistance in Relation to Antibody Titers in the Recipients

Immune serum was transferred to non immunized animals to analyse whether the observed differences in the increases in resistance against the intraperitoneal *E coli* infection were related mainly to humoral factors Similar resistance was obtained after transfer of the serum as in the immunized animals Also immune serum at 1/8 dilution protected the recipients (Table 2), while heterologous immune serum did not protect the recipients (Table 3) Furthermore the immune serum raised in different strains seemed to be of similar protective efficacy indicating that antibody avidity within the ranges recorded was of little importance Thus immune serum raised in CBA mice given to C3H/HeJ mice resulted in a similar resistance as in the C3H/HeJ mice given immune serum from C3H/HeJ mice This was also the case when using immune sera from the A/J mice in CBA mice and vice versa (Table 2) Thus compared with untreated mice of homologues strain the relatively susceptible A/J and C3H/HeJ mice were more efficiently protected by the antibodies than were the more resistant CBA mice (cf Table 1 and 2)

Relation between Dose for Immunization Resistance to Infection and Antibody Titer

The relationship was established for the CBA and NMRI mice regarding the number of bacteria used for immunization and the rises of resistance and antibody titer Immunizing doses of 10^2 to 10^4 bacteria seemed sufficient to induce protection in the animals This trend was verified in repeated experiments The small antigen doses, however only caused little antibody formation in the animals particularly in the NMRI mice, as recorded with the sensitive ELISA technique despite the protection induced (results not shown)

Animals of the NMRI strain were immunized with different amounts of bacteria to obtain serum to be used in transfer experiments It was found that transfer of 0 4 ml serum containing 06 antibodies from animals immunized with 10^7, 10^4 or 10^3 *E coli* 06 bacteria provided similar protection of the recipients despite that very little antibodies could be recorded in their serum (Table 3) Furthermore the serum from the animals immunized with 10^7 bacteria could be diluted 16 fold without losing its protective activity (Table 3), while higher serum dilutions in other experiments did not give any protection (results not shown) Control serum raised against serologically heterologous 015 bacteria did not provide any protection (Table 3)

To check whether the noted increases in resistance were not due to an unspecific local inflammatory reaction in the peritoneum after the injections injections with an antigenically different strain *E coli* 015 were compared with that obtained with

TABLE 3 *Dilution of Bacterial Suspension for LD_{50} and Titers of 06 antibodies in NMRI Mice after Transfer of Diluted Immune Serum Raised against Varying Doses of* E coli 06 K13

Group	Transfer of serum raised against	Serum dilution	Dilution of bacterial suspension for LD_{50} IgM	Antibody titer in recipient IgM	
1	10^7 06 bacteria	undil	1/10[a]	48	34
2	10^7 06 bacteria	1/4	1/10[a]	27	10
3	10 06 bacteria	1/16	1/15[a]	<10	<10
4	10^4 06 bacteria	undil	1/10[a]	18	18
5	10^3 06 bacteria	undil	1/15[a]	12	<10
6	none		1/60	<10	<10
7	10^7 015 bacteria	undil	1/50	<10	<10

a) = Significantly different from group 6 and 7

TABLE 4 *Effect of the Route of Stimulation on the Rise of Resistance in NMRI Mice*

Group No	Immunizing *E coli* strain	Route for immunization	Interval between immunization and infection	Dilution of bacterial suspension for LD50
1	06 K13	i p	4	1/5[a]
2		i v	4	1/5[a]
3	015 K?	i p	4	1/15[b]
4		i v	4	1/10[b]
5	015 K?	i p	1	1/1[a]
6		i v	1	1/10[b]
7	None			1/50

a) = Significantly different from groups 3 4 6 7
b) = Significantly different from group 7

homologous 06 bacteria Such treatment of the animals induced protection of similar magnitude regardless whether given by the i p or i v route 4 days prior to the challenge while given one day prior to challenge the injection by the i p route induced much better protection than that by the i v route (Table 4) When immune serum raised against heterologous 015 bacteria was administered i p one day prior to infection no protective effect could be recorded (Table 3)

DISCUSSION

This investigation revealed that mice of different inbred strains differ a great deal in their susceptibility to systemic *E coli* 06 infection The genetic pattern does not agree to what has been repeated for resistance against *Salmonella typhimurium* in mice Thus A/J CBA and C3H mice have been found resistant against Salmonella while C57 Bl mice were susceptible (8–10 19) Furthermore the results demonstrate that such strain related resistance cannot be referred to the genetically determined endotoxin resistance (14 20) Thus the C3H/HeJ mice most resistant to the endotoxin were more suscept[illegible] mice [illegible] endo[illegible] back ground did not show any increased resistance against intraperitoneal *E coli* infection compared to their nu/ + litter mates according to the present results The nu/nu mice have been reported to compensate for their thymic deficiency i a by a higher NK cell activity and also to be more resistant to various types of infections than their nu/ + litter mates (17) Consequently in view of the present results such compensating defence factors of the nu/nu mice have little effect on the intraperitoneal *E coli* infection utilized in the present investigation

Macrophages and polymorphonuclear leukocytes may be of vital importance in the defence against *E coli* bacteria by phagocytosis of bacteria and neutralization of endotoxin (4) The phagocytic function of the macrophages in mice of different strains has previously been related to the ability of the animals to form antibodies of high avidity against protein antigens Thus C3H mice formed antibodies of higher avidity than did for instance CBA mice (15) In view of these findings the present results with C3H mice might suggest that in non immunized mice the macrophages are poorly effective in the defence against systemic *E coli* infection. This is also supported by *in vitro* experiments with a bactericidal phagocytosis system (to be published) as well as the noted susceptibility of non immunized but endotoxin resistant C3H/HeJ mice In the latter animals inflammatory reactions to endotoxin have been reported to be dominated by mononuclear cells while in animals more susceptible to the endotoxin the inflammatory reactions are dominated by polymorphonuclear cells (14) [illegible] 06 antigen

Immunization of the mice with *E coli* 06 bacteria with homologous 0 antigen to those used for infection resulted in enhanced resistance to infection in all strains Similar increase of the resistance was found in CBA nu/nu nu/ + and NMRI mice despite that the animals of nu/nu and NMRI strains formed the smallest antibody amounts This was

explained by the transfer experiments showing that minute amounts of antibodies were sufficient for protection The presence of functioning thymus derived lymphocytes may be necessary for the antibody response to develop against the *E coli* bacteria although the thymus-derived cells may not be necessary for the development of defence factors against *E coli* infection, since the nu/nu mice formed less antibodies than the nu/+ mice but were similarly protected This suggests another mechanism for resistance against the *E coli* infection than against *S typhimurium* infection since T cell mediated delayed type hypersensitivity has been indicated to be important against the latter (19)

The results in the C3H/HeJ mice indicated that high avidity antibodies together with mononuclear inflammatory cells were as efficient in the defence against the bacteria in the C3H/HeJ mice as were antibodies of lower avidities and polymorpho nuclear cells in the other strains (14) In the experi ments where transfer of serum was utilized even antibodies at undetectable low levels seemed protec tive This corresponds to a recent report suggesting efficient protective function by »natural antibodies« (7) In agreement with that report (7) but in contrast to others (21 22) antibodies against the Re structure did not seem to be involved in the protection against the *E coli* infection It may be possible however that in case of liberated endotoxin such Re antibodies can neutralize the endotoxic effect which may aid in protection against endotoxin shock

Since various mice strains including the CBA and C3H/HeJ mice have been reported to have comple ment factors of similar efficacy (14) the contribution of the complement factors for the defence in the employed system although very important (unpub lished results) does not explain the differences in susceptibility between the various mice strains

In conclusion in the test system employed working with 0 antibodies only (3 13) high antibody levels do not seem to provide better protection to *E coli* bacteremia induced by the i p infection than do low levels The results in the present investigation should be compared with the clinically well known efficient protection by the administration of small amounts of antibodies in commercial gammaglobulin preparations The mi nute amounts of antibodies may work in concert with other defence systems e g complement factors and phagocytic cells and be particularly efficient in susceptible individuals (1) The present findings which correspond to other data from this laboratory concerning drug immunosuppressed mice (1) may also give further support to the view of the contribution of low levels of »natural« antibodies to the bactericidal effect of normal serum (reviewed by *Olling* 16)

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SERUM BACTERICIDAL EFFECT ON CAPSULATED AND NON-CAPSULATED *HAEMOPHILUS INFLUENZAE*

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Branefors P & Dahlberg T Serum bactericidal effect on capsulated and non-capsulated *Haemophilus influen ae* Acta path microbiol scand Sect. C 88 47–56 1980

In order to test serum bactericidal activity on *Haemophilus influen ae* a micromethod was employed The bactericidal (BC) effect of preimmune and hyperimmune rabbit sera was studied on a type a and a type b strain and their respective non-capsulated variants The influence of variation of the experimental conditions on the BC effect was investigated It was observed that the amount of guinea pig complement added as well as the number of bacteria and reaction time had an influence on the BC effect The serum dilution showing ≥50% killing of the inoculum was chosen as endpoint titre (BC titre) Unheated preimmune rabbit sera in which no specific antibodies were demonstrable with the complement fixation assay were bactericidal for the two non-capsulated strains Heat treatment to 56 °C for 5 min destroyed this effect, which could not be restored by addition of diluted guinea pig serum Unheated preimmune rabbit sera were not bactericidal for capsulated bacteria Hyperimmune sera showed heat resistant (56 °C 30 min) BC activity on both non-capsulated and capsulated bacteria the latter showing quite high BC titres (about 1 10³) Serum samples form 30 blood donors [illegible]

[illegible] these strains being more resistant than the two non capsulated variant strains utilized in the study

Key words Serum bactericidal effect *Haemophilus influen ae* capsulated non-capsulated unheated heated serum

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In *1932 Ward & Wright (27)* reported that a bactericidal effect could be produced on capsulated *H influenzae* bacteria by fresh human serum from adults or by a horse anti *H influenzae* serum + complement from guinea pig serum In 1933 *Fothergill & Wright* (12) studying the bactericidal effect against *H* [illegible]

[illegible] e occurrence of *H influenzae* meningitis to be inversely related to the bacterial activity demonstrated in the same ages The bactericidal activity demonstrated has later been suggested to be due to antibodies directed against the capsular substance (1 26) Some recent studies reporting bactericidal activity in sera of individuals immunized with purified capsular substance (PRP) support the suggestion that anti-capsular antibodies have a bactericidal effect (2 24) However the observation that such sera may show bactericidal activity even after absorption with PRP indicates that additional factors assumed to be antibodies against somatic antigens may be of importance for the bactericidal effect of certain sera against capsulated *H influenzae* (2 18)

Non-capsulated *H influenzae* bacteria are gene

rally more sensitive to serum bactericidal activity than capsulated bacteria (2, 12) However non-capsulated *H influenzae* might show resistance to serum bactericidal activity as shown in the study by *Gump et al* in 1971 (13) He found that 21 out of 130 sera from healthy individuals lacked a bactericidal effect on the non-capsulated strain used whereas only 1 of these sera lacked such an effect against the *H influenzae* type b strain

In recent years most studies concerning the bactericidal effect of sera on *H influenzae* have investigated human sera and only in a few of these have limited studies of sera from animal experiments been included (2, 15, 18, 24) The results of these studies on animals were in accordance with those on human sera, except that it was found that various laboratory animals in contrast to humans, did not respond with detectable antibodies after immunization with PRP (2)

In the aforementioned studies there has been considerable variation in the bactericidal assays employed different bacterial strains different amount of bacteria, and different sources of complement and undiluted – diluted unheated – heated sera, etc The results of different investigations are therefore not directly comparable

The aim of the present investigation was to study what influence variation in the experimental conditions may exert on the bactericidal activity (titre) of a serum The BC effects of rabbit preimmune and hyperimmune sera were studied on capsulated and non capsulated *H influenzae* by means of a micromethod (3) A number of blood donors sera were used for examining the applicability of the test to human sera A further purpose was to study possible differences in the susceptibility of various newly isolated non capsulated strains to the bactericidal activity of rabbit preimmune sera

MATERIALS AND METHODS

Strains

H influenzae type a strain Smith and *H influenzae* type b strain RAB and their respective non capsulated variants were used in the study The designations were Ma Mb and Sa Sb respectively The capsular antigen and the 0 antigen of the strains have previously been investigated by means of gel diffusion techniques (6 7) Ten additional non capsulated *H influenzae* strains newly isolated from patients with various infections were used in part of the study

Rabbit Sera

For each of the strains Ma Mb Sa and Sb two rabbits were immunized intravenously with bacterial suspensions The medium and the cultivation techniques have been described previously (5 6) All the eight rabbits were bled before the immunizations were initiated so as to obtain preimmune sera Each rabbit was given one primary immunization dose with about 10^8 bacteria and one month later a booster immunization with the same amount After another three weeks a hyperimmunization course was started and about 9 immunizations were given within a period of 6–8 weeks The bacterial amount was increased in twofold steps from 10^8 to $6 \cdot 10^9$ bacteria and in total each rabbit was given $2–4 \cdot 10^{10}$ bacteria One week after the last injection the final bleeding was performed by means of heart puncture The sera thus obtained were dispensed in 1–2 ml aliquots and kept at −70 °C until used

Additional rabbit serum samples from non immunized animals were utilized for the study of bactericidal activity of rabbit preimmune sera on fresh *H influenzae* isolates

Human Sera

Sera from 30 blood donors were tested for bactericidal activity on Ma and Mb as well as on the Sa and Sb strains

Bactericidal Assay

A modification of the bactericidal method described by *Anderson et al* (3) was employed The bactericidal (BC) tests were performed on Linbro Disposo Trays (IS FB 96) well volume 0 4 ml (Instrumentfirman Labora AB Stockholm Sweden)

Complement Pooled guinea pig serum stored at −70 °C in 1 ml aliquots for up to 3 months was used as complement source in all experiments except when otherwise stated The BC effect on the strains of each new batch of guinea pig serum was titrated In tests with Ma and Mb bacteria the guinea pig serum was used undiluted or diluted 1 2 while in tests with Sa and Sb bacteria it had to be diluted 1 20 to 1 30

Sera The rabbit and human sera were tested unheated or heated to 50–56 °C for 5–30 minutes

Bacterial suspensions From an overnight culture on solid AFH medium (5) one loopful was inoculated into 25 ml fluid AFH medium and incubated for 3–4 hours at 37 °C on a Gyrotyry shaker to a density of about $1–3 \cdot 10^9$ bacteria per ml The cultures were then chilled and diluted to an optical density of 0 4 in a Beckman C colorimeter (at 660 mm) which corresponds to about $1–2 \cdot 10^9$ bacteria per ml The diluent consisted of cold autoclaved AFH medium with no hematin added but with additional $CaCl_2$ 0 00045 M and $MgCl_2$ 0 015 M Further dilutions of the culture were performed with the same diluent in order to obtain from about $2 \cdot 10^2$ to $2 \cdot 10^6$ bacteria per 0 025 ml in the tests Autoclaved AFH medium was used as diluent for the tests since preliminary studies had shown that it permitted no or only a slight increase in the number of bacteria when incubated at 37 °C for 1–3 hours with addition of diluted guinea pig serum but without test serum added whereas [illegible] physiological saline

performed in 0.025 ml PBS on the Linbro microtiter trays. After that 0.025 ml guinea pig serum (appropriately diluted) and 0.025 ml of diluted bacterial suspension were added. The trays were closed and incubated for 15 min to 3 h at 37 °C on the Gyrotory shaker. This time is henceforth referred to as »reaction time«. After reaction time about 0.25 ml AFH agar medium containing 2% agar was added. After 18–24 h of incubation at 37 °C individual colonies were readily visible in the agar with the aid of a magnifying glass. In each experiment two sets of controls consisting of 100%, 50%, 25% and 12% of the bacterial inoculum suspended in AFH medium and diluted guinea pig serum were included. To one of the control sets AFH agar medium was added immediately, to the other it was added after reaction time.

The bactericidal activity of a serum to be tested was calculated either by counting the number of colonies in the wells or, in case of a large number of colonies, by comparison with the control sets. The serum dilution which showed a reduction ≥50% of the number of colonies was chosen as the endpoint titre (BC titre). The reproducibility of the test was ± one titre step.

Hemolytic indicator system. A suspension of 1.5% sheep erythrocytes sensitized with rabbit anti sheep erythrocyte serum was used for testing complement consumption in some of the BC experiments where prozone phenomena were observed. Sensitized sheep erythrocytes 0.5 ml were added to each well after reaction time and the tests were incubated at 37 °C for another 45 minute and after that possible lysis of the erythrocytes was registered.

Anti capsular and Anti cell Wall Antibody Assays

Indirect hemagglutination assay (IHA) was used to measure antibodies against the capsular antigens of Ma and Mb bacteria and the complement fixation assay (CF) to measure antibodies against the cell wall antigens by means of bacterial suspensions from Sa or Sb. The techniques employed have previously been described (8).

EXPERIMENTS AND RESULTS

Methodological Aspects

A series of experiments were performed in order to study various factors which might have an influence on the results of the BC assay and to determine the conditions under which the assay would be best suited for the detection of bactericidal activity of serum. The *amount of guinea pig serum*, the *inoculum size* and the *reaction time* were varied one by one in assays performed according to the principles accounted for in Materials and Methods.

Influence of various amounts of complement on bactericidal effect. The guinea pig sera were found to be bactericidal for both Sa and Sb bacteria, bactericidal activity being observed in dilutions 1:4–1:8 and 1:8–1:16, respectively (Table I). To ascertain that no BC effect was caused by the added guinea pig serum per se, and that the serum contained enough complement for the expression of a maximal BC titre of preimmune sera on Sa and Sb

TABLE I. *Bactericidal Effect on Sa Bacteria of Fresh Guinea Pig Serum and a Rabbit Preimmune Serum (Unheated and Heated 56 °C for 5 min) in an Experiment with an Inoculum of 240 Bacteria per Well and a Reaction Time of 2 Hours at 37 °C*

Sum tested	Percent of surviving Sa bacteria in serum diluted							
	1/2	1/4	1/8	1/16	1/32	1/64	1/28	1/256
Guinea pig serum diluted in PBS	0	0	<1[a]	100	100	100	100	100
Rabbit serum (unheated) diluted in PBS – guinea pig serum	0	0	0	<1	50	100	100	100
Rabbit serum (heated) diluted in PBS – guinea pig serum	100	100	100	100	100	100	100	100
Rabbit serum (unheated) + guinea pig serum diluted 1/20	0	0	0	0	0	<1	50	100
Rabbit serum (heated) + guinea pig serum diluted 1/20	100	100	100	100	100	100	100	100

[a]) The BC titre underlined

TABLE 2 *Effects of Different Amounts of Guinea Pig Serum on the Bactericidal Effect of an Anti-Ma Hyperimmune Serum Inoculum 150 Bacteria/Well Reaction Time 2 Hours*

ɔncentra-n of ded ınea pıg ʼum	Percent of surviving Ma bacteria in serum diluted											
	1/128	1/256	1/512	1/1024	1/2048	1/4100	1/8200	1/16400	1/32800	1/65500	1/131100	1/262200
1/1	100	50[a]	20	10	<1	0	<1	0	0	0	2[b]	100
1/2	100	100	100	100	100	50[a]	12	15	2	10	3[b]	100
1/4	100	100	100	100	100	100	100	100	100	100	100	100

[a]) The prozone titre
[b]) The BC titre

bacteria, a range of dilutions – from 1 10–1 40 – were tested The results revealed that guinea pig serum diluted up to 1 30 and 1 40 for Sa and Sb, respectively, contained enough complement for the expression of a maximal BC titre of preimmune sera This amount of complement was also sufficient for a maximal BC titre of anti-Sa and anti Sb hyperimmune sera to be obtained However, in these sera a prozone phenomenon was observed

The guinea pig sera and preimmune rabbit sera were not in themselves bactericidal for Ma and Mb bacteria Therefore, hyperimmune sera were used in order to study suitable amounts of complement in assays with Ma and Mb bacteria The results showed that when the same dilution of guinea pig serum as for Sa and Sb bacteria was used as a source of complement no BC effect was registered For that reason undiluted guinea pig serum and dilutions of up to 1 6 were tested in studies of BC activity on capsulated bacteria Addition of guinea pig serum diluted 1 2 or less was necessary in order to obtain a demonstrable BC effect Table 2 gives the results of an experiment with Ma bacteria As may be seen, the larger amount of complement (undiluted guinea pig serum) did not increase the BC titre of the hyperimmune serum A prozone phenomenon was observed, being less pronounced with the addition of concentrated guinea pig serum

Influence of bacterial inoculum size on bactericidal effect A series of experiments were performed in order to study what effects variation of the bacterial inoculum size had on the BC titre of preimmune and hyperimmune sera A wide range of Sa and Sb concentrations between 1–2 10^2 and 1–10^6 cells, were tested with preimmune sera The results showed that tenfold increased inoculum size resulted in about twofold lower BC titres of the sera Fig 1 shows an experiment with Sa bacteria and a preimmune serum It is notable that with an inoculum of 2 10^6 Sa bacteria a few percent of the cells were not killed even by the serum dilution 1 2

When hyperimmune sera were tested with the same range of Sa and Sb bacteria it was noticed that a tenfold increased bacteria concentration resulted in less than twofold lower BC titres of serum except when an inoculum of about 2 10^5–2 10^6 bacteria/well tested (Fig 2) As may be seen in Fig 2, when about 2 10^6 bacteria/well were tested 100% killing of the bacteria was observed only in two

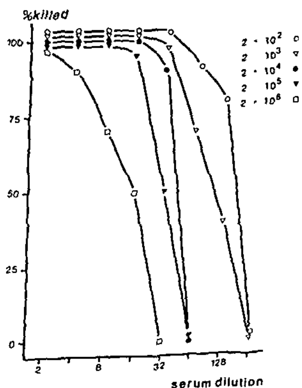
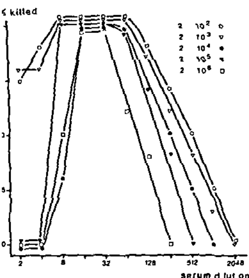


Fig 1 Effect of inoculum size on the bactericidal effect of unheated preimmune serum on Sa bacteria (reaction time 2 h)



ig 2 Effect of inoculum size on the bactericidal effect f homologous hyperimmune serum (heated 56 °C 30 min) on Sa bacteria (reaction time 2 h)

lutions of the hyperimmune serum due to a very marked prozone phenomenon

It was further observed that when more than 10^3 bacteria/well were used it was somewhat difficult to register the BC titre despite the use of the control sets Therefore in the tests with preimmune as well as hyperimmune sera 1–4 10^2 Sa and Sb bacteria were usually added to every well

In similar experiments with Ma and Mb bacteria it was observed that with an inoculum over about 2 10^3 bacteria/well it was impossible to register a BC titre because of confluent growth of the 100% control set Therefore the inoculum of Ma and Mb kept below 4 10^2 bacteria was found suitable for Ma and Mb too

Influence of reaction time on bactericidal effect In order to study the influence of the reaction time on BC titres of preimmune sera on Sa and Sb bacteria the reaction time was varied from 15 min to 3 h BC titres were demonstrable for both Sa and Sb after a reaction time of 15 min The titres increase with longer reaction time the increase being more pronounced for Sb than for Sa In Fig 3 the results of experiments with three preimmune sera are given

The experiments performed with anti-Sa and anti Sb hyperimmune sera showed that in order to obtain a demonstrable BC titre on Sa and Sb bacteria a reaction time of 30–60 min was necessary Fig 4 gives the results of an experiment

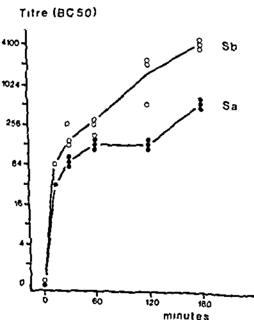


Fig 3 Effect of reaction time on the BC titre of three unheated preimmune sera on Sa and Sb bacteria (about 300 bacteria/well)

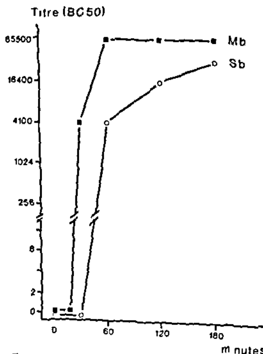


Fig 4 Effect of reaction time on the BC titre of homologous hyperimmune serum (heated 56 °C 30 min) in experiments on Mb and Sb bacteria (about 360 and 180 bacteria/well respectively)

TABLE 2 *Effects of Different Amounts of Guinea Pig Serum on the Bactericidal Effect of an Anti-Ma Hyperimmune Serum Inoculum 150 Bacteria/Well Reaction Time 2 Hours*

Concentration of added guinea pig serum	Percent of surviving Ma bacteria in serum diluted											
	1/128	1/256	1/512	1/1024	1/2048	1/4100	1/8200	1/16400	1/32800	1/65500	1/131100	1/262200
1/1	100	50[a]	20	10	<1	0	<1	0	0	0	2[b]	100
1/2	100	100	100	100	100	50[a]	12	15	2	10	3[b]	100
1/4	100	100	100	100	100	100	100	100	100	100	100	100

a) The prozone titre
b) The BC titre

bacteria, a range of dilutions – from 1 10–1 40 – were tested The results revealed that guinea pig serum diluted up to 1 30 and 1 40 for Sa and Sb, respectively, contained enough complement for the expression of a maximal BC titre of preimmune sera This amount of complement was also sufficient for a maximal BC titre of anti-Sa and anti-Sb hyperimmune sera to be obtained However, in these sera a prozone phenomenon was observed

The guinea pig sera and preimmune rabbit sera were not in themselves bactericidal for Ma and Mb bacteria Therefore, hyperimmune sera were used in order to study suitable amounts of complement in assays with Ma and Mb bacteria The results showed that when the same dilution of guinea pig serum as for Sa and Sb bacteria was used as a source of complement no BC effect was registered For that reason undiluted guinea pig serum and dilutions of up to 1 6 were tested in studies of BC activity on capsulated bacteria Addition of guinea pig serum diluted 1 2 or less was necessary in order to obtain a demonstrable BC effect Table 2 gives the results of an experiment with Ma bacteria As may be seen, the larger amount of complement (undiluted guinea pig serum) did not increase the BC titre of the hyperimmune serum A prozone phenomenon was observed being less pronounced with the addition of concentrated guinea pig serum

Influence of bacterial inoculum size on bactericidal effect A series of experiments were performed in order to study what effects variation of the bacterial inoculum size had on the BC titre of preimmune and hyperimmune sera A wide range of Sa and Sb concentrations between 1–2 10^2 and 1– 10^6 cells, were tested with preimmune sera The results showed that tenfold increased inoculum size resulted in about twofold lower BC titres of the sera Fig 1 shows an experiment with Sa bacteria and a preimmune serum It is notable that with an inoculum of 2 10^6 Sa bacteria a few percent of the cells were not killed even by the serum dilution 1 2

When hyperimmune sera were tested with the same range of Sa and Sb bacteria, it was noticed that a tenfold increased bacteria concentration resulted in less than twofold lower BC titres of serum, except when an inoculum of about 2 10^5–2 10^6 bacteria/well tested (Fig 2) As may be seen in Fig 2, when about 2 10^6 bacteria/well were tested, 100% killing of the bacteria was observed only in two

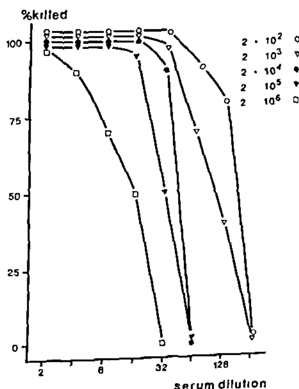


Fig 1 Effect of inoculum size on the bactericidal effect of unheated preimmune serum on Sa bacteria (reaction time 2 h)

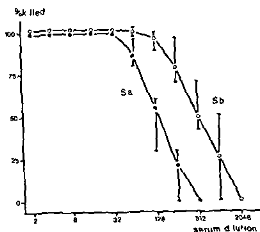


Fig 5 Bactericidal effect of eight unheated preimmune rabbit sera on Sa and Sb bacteria in various serum d lutions with a reaction time of 2 h and an inoculum per well of about 350 Sa and 250 Sb bacteria respectively

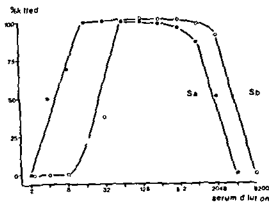


Fig 6 Bactericidal effect of homologous hyperimmune sera (heatet 56 °C 30 min) on Sa and Sb bacteria with a reaction time of 2 h and an inoculum per well of about 200 bacteria

Bactericidal Activity of Rabbit Hyperimmune Sera

The anti Sa and anti Sb hyperimmune sera had CF titres of about 2 10^4 and 5 10^4 respectively The anti Ma and anti Mb hyperimmune sera had IHA titres of about 1 10^4 and 2 10^4 and CF titres of about 2 10^3 and 3 10^4 respectively

The BC titres of unheated anti Sa and anti Sb hyperimmune sera were about sixteenfold higher than those of preimmune sera When an inoculum of about 200–300 bacteria and a reaction time of 2 h were used the anti Sa hyperimmune sera had BC titres of 1 2 048–1 4 100 and the anti Sb BC titres of 1 4 100–1 8 200 (Fig 6)

In contrast to the BC titres of the preimmune sera the titres of the anti Sa and anti Sb hyperimmune sera remained unchanced or decreased at most one twofold dilution after heat treatment 56 °C for 30 min however the prozone were more marked

In experiments with the two anti Ma and two anti Mb hyperimmune sera very high titres were obtained the anti Ma titres being about 1 10^5 and the anti Mb titres about 1–2 10^5 when an inoculum of about 200 bacteria and a reaction time of 2 h were used These titres were unchanged or reduced one twofold dilution by heat treatment of the sera 56 °C for 30 min the prozones however were more marked

Bactercidal Activity of Human Sera

Fresh sera from 30 blood donors were tested for bactericidal effect on the Ma Mb Sa and Sb bacteria The serum samples were tested unheated and heated to 56 °C for 5 and 30 min The results showed that all the unheated human sera had a BC effect on [illegible]

heating time of the sera was increased to 30 min the BC titres remained unchanged or decreased one titre step The Sa bacteria were more resistant to the

TABLE 3 *Bacterial Effects (BC Titre) of 30 Human Sera from Blood Donors on the Ma Mb Sa and Sb Bacteria React on Time 2 Hours*

	Serum dilution																	
	Unheated serum									Serum heated 56 °C 5 min								
cteria	<1	1	2	4	8	18	32	64	128	<1	1	2	4	8	16	32	64	12
a		10	12	7	1					2	7	14	5	2				
b			3	7	7	12		1			2	10	10	7	1			
.	11	9	7	3						17	6	4	3					
)					2	3	5	12	8	15	2	1	3	3	3	2	1	

with a hyperimmune serum against Sb bacteria A BC titre of 1 4,100 was observed after a reaction time of 60 min, the titre increasing slowly during further reaction time

When the anti-Ma and anti-Mb hyperimmune sera were tested against homologous Ma and Mb bacteria and guinea pig serum diluted 1 2, a BC titre was observed after a reaction time of 30 min The BC titres increased during further reaction time, reaching a maximum after 60–90 min (Fig 4)

Influence of the amount of complement, inoculum size and reaction time on the prozone phenomenon Prozones were registered for all hyperimmune sera being most pronounced in the two anti-Sb and the two anti-Ma sera Experiments were performed in which the influence of the amount of complement, the inoculum size and the reaction time on the prozone phenomenon was registered The amount of complement added was found to be important for the extent of the prozone When inoculum size and realation time were kept constant increased amounts of complement diminished the prozone titre (titre $\geq 50\%$ survival of the inoculum) of all hyperimmune sera As may be seen from Table 2, the anti Ma hyperimmune serum tested with guinea pig serum diluted 1 2 showed a prozone titre of no less than 1 4,100, when undiluted guinea pig serum was added, the prozone titre diminished to 1 256

In order to study to what extent the complement had been consumed during reaction time, a hemolytic indicator system was added to each well after 2 hours It was revealed that all complement had been consumed in the serum dilutions giving a prozone phenomenon while in the higher dilutions 100% hemolysis was observed as an indication of the presence of excess complement No anti-complementary activity was demonstrable in the hyperimmune serum samples diluted 1 2 nor in the suspensions of capsulated or non-capsulated bacteria

Influence of the Sa and Sb inoculum size on the prozone phenomenon was observed too Keeping the amount of complement and reaction time constant the prozone was more extended when the inoculum was increased The tests with anti-Sa hyperimmune sera revealed that when 10^2–10^3 bacteria/well were added no prozone titre was observed A larger inoculum size (about 10^4–10^6 bacteria) caused a prozone titre of about 1 8 (Fig 2) The two anti-Sb hyperimmune sera showed more extended prozones than the anti Sa sera In these sera the prozone titres were observed even with as low an inoculum as about 10^2 bacteria (titre about 1 516)

Influence of reaction time on the prozone phenomenon was demonstrable for all the hyperimmune sera The prozone titres diminished with increasing reaction time for the anti Ma and anti Mb sera as well as for anti-Sa and anti Sb sera The anti-Ma and anti-Mb sera and the anti-Sa and anti Sb sera showed minimal prozone after a reaction time of 90 min and 120 min respectively The longer reaction time did not further diminish the prozone

Reproducibility of the BC test In order to evaluate the accuracy of the test, the BC titres of 12 titrations of anti-Mb hyperimmune serum performed on different occasions with different batches of guinea pig serum, using a reaction time of 2 h and inoculum size of 1–4 10^2 bacteria were registered The results showed that on 2 occasions the titre 1 65,500 was registered, whereas on 7 occasions the titre was 1 131,000 and on 3 occasions 1 262,200 It may be noted that all BC titres fell within an interval corresponding to two dilution steps

Bactericidal Activity of Rabbit Preimmune Sera

The preimmune sera used in the studies had no demonstrable antibodies against the capsular antigen (IHA) and against the 0 antigens (CF) In experiments with unheated preimmune sera no obvious BC effect was demonstrable on Ma or Mb bacteria even when undiluted guinea pig serum was added as the source of additional complement however, in some experiments a slight reduction of the number of colones (<50% reduction) was observed when undiluted rabbit serum was used In contrast to these results the unheated preimmune serum samples from all the eight rabbits had a BC effect on Sa and Sb bacteria This BC effect was demonstrable without the addition of guinea pig complement The BC titres, however increased four- to eightfold when diluted guinea pig serum was added (Table 1) Approximately the same degree of BC activity was registered for the eight preimmune sera the difference not exceeding one titre step between the different sera The BC titres for Sa were 1 64–1 128 and for Sb 1 256–1 512 when a reaction time of 2 h and an inoculum of about 200 bacteria were used (Fig 5)

In a series of experiments the heat lability of the preimmune serum BC effect was studied by heating the sera to 50 °C and 56 °C for 5 to 30 min A marked heat lability of the BC effect on preimmune sera was observed heating the sera to 50 °C for 30 min reduced the BC titres to 1 4–1 8 of that of the unheated serum Heating to 56 °C for as short a time as 5 min destroyed all BC effect of the preimmune sera

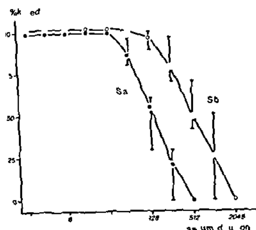


Fig 5 Bactericidal effect of eight unheated preimmune rabbit sera on Sa and Sb bacteria in various serum dilutions with a reaction time of 2 h and an inoculum per well of about 350 Sa and 250 Sb bacteria respectively

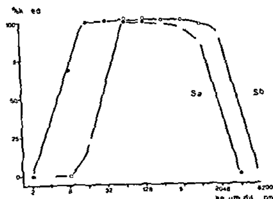


Fig 6 Bactericidal effect of homologous hyperimmune sera (heated 56 °C 30 min) on Sa and Sb bacteria with a reaction time of 2 h and an inoculum per well of about 200 bacteria

Bactericidal Activity of Rabbit Hyperimmune Sera

The anti Sa and anti-Sb hyperimmune sera had CF titres of about 2 10^4 and 5 10^4 respectively The anti Ma and anti Mb hyperimmune sera had IHA titres of about 1 10^4 and 2 10^4 and CF titres of about 2 10^3 and 3 10^4 respectively

The BC titres of unheated anti Sa and anti Sb hyperimmune sera were about sixteenfold higher than those of preimmune sera When an inoculum of about 200–300 bacteria and a reaction time of 2 h were used the anti Sa hyperimmune sera had BC titres of 1 2 048 1 4 100 and the anti Sb BC titres of 1 4 100–1 8 200 (Fig 6)

In contrast to the BC titres of the preimmune sera the titres of the anti-Sa and anti Sb hyperimmune sera remained unchanged or decreased at most one twofold dilution after heat treatment 56 °C for 30 min however the prozone were more marked

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Bactericidal Activity of Human Sera

Fresh sera from 30 blood donors were tested for bactericidal effect on the Ma, Mb Sa and Sb bacteria The serum samples were tested unheated and heated to 56 °C for 5 and 30 min The results showed that all the unheated human sera had a BC effect on the capsulated Ma and Mb bacteria the titres being only slightly or moderately lowered by heating to 56 °C for 5 min (Table 3) When the heating time of the sera was increased to 30 min the BC titres remained unchanged or decreased one titre step The Sa bacteria were more resistant to the

TABLE 3 *Bactericidal Effects (BC Titre) of 30 Human Sera from Blood Donors on the Ma Mb Sa and Sb Bacteria Reaction Time 2 Hours*

	Serum dilution																	
	Unheated serum									Serum heated 56 °C 5 min								
Bacteria	<1	1	2	4	8	18	32	64	128	<1	1	2	4	8	16	32	64	128
Ma		10	12	7	1					2	7	14	5	2				
Mb			3	7	7	12		1			2	10	10	7	1			
Sa	11	9	7	3						17	6	4	3					
Sb					2	3	5	12	8	15	2	1	3	3	3	2	1	

TABLE 4 *Bactericidal Titres of Rabbit Preimmune Sera on Non capsulated* H influenzae *Newly Isolated from Patients Reaction Time 2 hours*

Strain number	Source	Clinical diagnosis	Bactericidal titre of rabbit sera[a)]
1	blood	septic abortion	16–32
2	blood	foetal sepsis	2–4
3	pus	genital abscess	32–64
4	epiglottis	epiglottitis	1–2
5	nose	sinusitis	32
6	nasopharynx	acute otitis media	2–4
7	nasopharynx	acute otitis media	4–8
8	nasopharynx	acute otitis media	1–2
9	nasopharynx	acute otitis media	16–32
10	nasopharynx	acute otitis media	4–8

a) The BC titre of individual serum samples varied one twofold dilution

BC effect of the human sera than was the parent Ma strain while Sb bacteria were considerably more sensitive than the parent Mb strain (Table 3) Heating the sera to 56 °C for 5 min caused decrease of BC activity against Sa bacteria in only about one third of the serum samples while BC activity against Sb bacteria decreased in all sera except two (titres 1 32 and 1 64) In half of the serum samples no BC activity was registered When the heating time was increased to 30 min the BC titres of the sera did not further decrease or were at most one titre step lower

Bactericidal Effect of Rabbit Preimmune Sera on fresh H influenzae *Isolates*

The BC effect of 10 rabbit preimmune sera on 10 non capsulated *H influenzae* strains isolated from various sources of infected patients was investigated Table 4 summarizes the results and as may be seen the strains differed a great deal in sensitivity to the BC factors present in normal rabbit sera their sensitivity ranging from titre 1 2 to 1 64 The two most resistant strains were isolates from a case of acute epiglottitis and a case of acute otitis media There was a connection between strain 2 and strain 5 the more resistant strain having been isolated from the blood of a new born premature child with intrauterine sepsis and the sensitive strain from the nose of the mother who at the time of delivery had acute sinusitis It seems notable that the non capsulated patient strains were less sensitive to the bactericidal effect of normal rabbit sera than were the non capsulated Sa and Sb bactericidal variants

DISCUSSION

The results of the present study indicate that a complement-dependent bactericidal (BC) effect might be caused on both capsulated and non capsulated *Haemophilus influenzae* bacteria by sera containing specific antibodies In addition non capsulated *H influenzae* bacteria might be sensitive to bactericidal factors in normal rabbit (preimmune) in which no antibodies against the tested bacteria were detectable

The result of the study on rabbit sera indicated that the three parameters studied complement concentration inoculum size and reaction time had an influence on the BC titres of preimmune as well as hyperimmune sera

Most workers have used a human serum as the source of additional complement (3 10 13 19) In our studies guinea pig serum was found to be a suitable source of complement and was thus used in the assays It was practicable to add guinea pig serum in a dilution 1 20 and 1 30 which did not cause a BC effect per se on non-capsulated bacteria but still contained sufficient complement for a BC effect of rabbit hyperimmune sera to appear

In papers presenting bactericidal tests on *H influenzae* there have been great variations in inoculum size from about 100 to about 2 10^5 bacteria (2 3 10 15 18 19, 24) We found that variation in the amount of bacterial inoculum had a comparatively moderate influence on the BC titre but with an inoculum size as low as 100–400 bacteria a killing effect of about 50% was easy to observe prozone phenomena (17) being less likely to be pronounced

Reaction time was one of the important factors

which may have an influence on the BC titres of the sera. The kinetics of the BC effect were different in preimmune and hyperimmune sera the latter needing a longer reaction time Therefore two hours seemed advisable and has been used for the main part of the BC assays on immune as well as preimmune sera

Although the prozone problem (17) was not studied in detail it was revealed by the addition of a hemolytic indicator system that all complement had been fixed in the region of the prozone while an excess of complement was present in the higher serum dilutions It seems probable that the inhibition of the BC effect was due to an excess of complement fixing antibodies operating at an ineffective site as proposed by *Muschel et al* (16) and *Norman* (20) rather than to non-complement fixing antibodies (IgA) constituting a steric hindrance to the action of the complement as proposed by *Rowley* (23) We found like others (16 20) that heat treatment of the hyperimmune sera increased the prozone titre possibly caused by an anticomplementary effect due to aggregation of serum proteins (14 16)

Only non-capsulated *H influenzae* bacteria were found to be sensitive to bactericidal factors in preimmune rabbit sera in which no antibodies against the bacteria tested were detectable The BC effect of normal sera on certain strains of Gram negative bacteria i e *E coli* is well known and has been attributed to activation of the alternative pathway (4 25) We found the factors causing the BC activity of the preimmune sera to be heat labile i e they were destroyed between 50–56 °C and absorbed by zymozan at 15 °C (unpublished results) These findings are well accordance with properties of the factors of the properdin system (4 21 22) Heating rabbit and human sera to 56 °C for 5 min might be a means by which the presence of specific bactericidal antibodies can be distinguished from BC activity of preimmune sera

The capsulated bacteria were not sensitive to bacterial activity of preimmune rabbit sera The results of our studies were agreement with an investigation on human sera in which it was found that capsulated *H influenzae* appeared to lack the ability to activate the complement through the alternative pathway in contrast to the *E coli* strain used (11) However our studies of the BC activity of hyperimmune sera indicated that capsulated *H influenzae* bacteria are very sensitive to complement activated through the classical pathway as the BC titre of the anti Ma and anti Mb sera was found to be quite high (about 1–2 5)

The method developed on rabbit sera was found to be suitable for ... its on human sera too The results indicate that normal human sera from adults to a great extent have antibodies against capsulated *H influenzae* type a and b Demonstrable antibodies against surface structures (0 antigen and/or proteins) of the two non-capsulated strains were less prevalent in the human sera tested

The fresh isolates of non-capsulated *H influenzae* from infected patients were like Sa and Sb sensitive to the BC activity of rabbit preimmune sera However the BC titres of the patient strains were all lower than for the Sa and Sb strains and moreover the sensitivity of the strains varied a great deal from 1 1–2 to 1 32–64 One possible reason for the difference in sensitivity of the patient strains as well as that of Sa and Sb respectively may be that the cell walls of non-capsulated bacteria differ as to the permeability of the activated complement. Permeability has been found to be of importance for the sensitivity to the BC effect of complement (23) Another possibility is that the outer membrane of different strains may possess variable amounts of proteins and LPS (9), which may affect the degree of complement activation through the alternative pathway This difference in sensitivity to serum BC effect may reflect a difference in virulence (e g invasiveness) among the strains as well

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STUDIES ON Co^b (COLTON) ANTIGEN AND ANTI-Co^b

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Jerne D & Lylloff K. Studies on Co^b (Colton) antigen and anti-Co^b Acta path microbiol scand Sect. C 88 57–60 1980

Using a reliable and strong reacting antiserum of the rare anti-Co^b specificity it was shown that the Co^b antigen was fully developed at birth Gene dosage effect could not be demonstrated The frequency of Co(b+) persons in the Danish population (7 8 %) was found not to differ from other Caucasian populations The antibody belonged to immunoglobulin class IgG without ability of fixing complement *in vitro*

Key words Co^b (Colton) antigen anti Co^b

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The introduction of the Colton blood group system of human red cells took place in 1967 by the description by *Heistø et al* (4) of an antibody defining the antigen Co^a Three years later *Giles et al* (3) reported the detection of an antibody reacting with the presumably antithetical allele product Co^b

Since then several other examples of anti-Co^a and anti-Co^b have been published (Table 3) but information characterizing especially the Co^b antigen and anti-Co^b is still to a great extent lacking Gene dosage effect on reaction strenght of the antigens development of antigen at birth and frequency of Co^b antigen in many populations Likewise serological characterization of anti-Co^b is still insufficient

As anti-Co^b is a rarely detected antibody it was decided to further characterize the Co^b-anti-Co^b system when a reliable and strong reacting anti Co^b became available

MATERIALS AND METHODS

Antisera

Anti Co^b (EGJ) was shown in a 60 years old male patient (A Rh positive Co(a+b–) Wr(a–)) suffering from myelofibrosis during 1 ½ years In this period he was transfused with 10 donorunits of erythrocytes – two of which were later shown to possess the Co^b antigen The anti Co^b in his serum was optimally reacting in saline followed by anti human globulin technique (AHG) and equally well by enzyme (2 step papain technique) followed by AHG With the enzyme technique an anti Wr^a (Wright blood group system) was furthermore found Blood was drawn from the patient at one occasion small aliquots of the serum were stored in liquid nitrogen and the needed amount thawed immediately prior to each investigation

The anti-Co^b (EGJ) showed 32 in titer by the saline + AHG technique

Anti Co^b (EP) was detected in a 60 years old female patient (A Rh positive Co(a+b–)) admitted in the [illegible]

[illegible] that is best by the saline or 2 step papain technique at 37 °C followed by AHG The patient died before the serological investigations were completed

Blood Samples

186 pairs of blood samples originating from mothers and their newborn children mostly sent to the blood bank for Rh prophylactive purposes were available for phenotyping and determination of Co^b antigen development. Only pairs of which both were ABO-compatible with the anti-Co^b (EGJ) were used for investigation Anti Wr^a in anti-Co^b testserum did not interfere as all Co(b+) persons included in this study were found Wr(a–) Along with these samples erythrocytes from 86 healthy blood donors were studied Erythrocytes were

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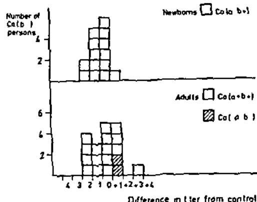


Fig 1 Antigenic strength of Co^b antigen on red blood cells from newborn children and adults compared to control Co(a + b +) adult red blood cells (see text)

Effect of Gene Dosage

Included in the determinations shown in Fig 1 are two samples found homozygous for the Co^b gene i e Co(a–b +) using anti-Co^a and anti Co^b for phenotyping One of these was found in this study the other originated from the collection of rare phenotype red cells stored in our blood bank On the basis of only these two examples no clear gene dosage effect was demonstrable

Frequency of the Co(b +) Phenotype

Among 186 mothers and 86 blood donors belonging to blood groups A and O 21 Co(b +) persons were found that means a frequency of Co(b +) persons in the Danish population amounting to 7 8% (95% confidence limits 4 8 – 11 4%)

DISCUSSION

The demonstration of anti-Co^b is a rare event The antibody is presumably *above all produced by* persons, who are good antibody responders Often therefore anti-Co^b is found in persons producing several other irregular antibodies stimulated by allotransfusion of erythrocytes (see Table 3) The *two anti-Co^b sera reported here are* remarkably pure and strong reacting The purity can be explained for the EGJ serum by the fact that EGJ expressed most of the strong blood group antigens himself (Fy^a c, D Jk^a) only lacking the K antigen However phenotype studies of the donors from whom he received red cells showed that he probably never had been exposed to this antigen

As the anti-Co^b is found to belong to the immunoglobulin class IgG and the Co^b antigen is fully developed at birth it can be anticipated that the antibody can pass placenta in pregnant women and provoke hemolytic disease of the newborn although this has not yet been reported The counterpart antibody anti-Co^a which shows similar immunological characteristics has caused hemolytic disease of the new born [illegible]

[illegible] reported for other populations of European descent (8)

TABLE 3 *Published Cases of Anti Co^b*

		Stimulation	Technique optimal for demonstration of anti-Co^b			Simultaneously occurring irregular antibodies
			Enzyme	Albumin	AHG	
Giles et al (3) 1970		Transfusion	+	+		Anti E Anti-C^w
Ikin et al (5) 1970	1)	Transfusion	+			Anti Fy^a Anti c
	2)	Transfusion	+			Anti-C Anti E Anti Fy^a
Lewis et al (6) 1971		?		?		Anti D Anti C Anti K Anti S
Case (1) 1971		Transfusion	+			None
Clausen (2) 1972		Transfusion	+			Anti Fy^a Anti S Anti E Anti Wr^a Anti I
Jerne et Lylloff (this report) 1979	1) EGJ	Transfusion	+	–	+	Anti Wr^a
	2) EP	Transfusion	+	–	+	None

TABLE 1 *Reaction Pattern of Anti Co^b Sensitized Red Blood Cells with Specific Anti Ig Sera*

Co(b +) red blood cells incubated 1 hour at 37 °C with	Anti IgG	IgA	Reaction with IgM	C_3/C_4	Broad spectrum AHG reagent
Fresh anti Co^b (EGJ)	+	–	–	–	+
Fresh anti Co^b (EP)	+	–	–	–	+

used within 1 week of sampling although pilot studies showed unaltered Co^b antigens after 5 weeks of storage in ACD anticoagulant solution

Serological Technique

Phenotyping Anti Co^b (EGJ) was used throughout this study and always diluted 1 1 in saline For phenotyping and titrations 5% suspensions of erythrocytes in saline were incubated with antiserum dilutions at 37 °C for one hour followed by AHG using a broad spectrum antiglobulin reagent The results were red with the naked eye in accordance to general serological principles Positive and negative controls were included

Titrations were carried out as two fold master titrations Using the same antiserum the endpoint of titration was taken as a measure of the strength of the antigen The same Co(a + b +) donor supplied control test cells for simultaneous titrations in every case for reasons of comparison

Classification of anti Co^b was carried out using commercial agglutinating classspecific anti Ig and anti complementsera Anti IgG –IgM – IgA and anti C_3/C_4 (Organon®) After incubating Co(b +) red blood cells with undiluted fresh anti Co^b for 60 minutes at 37 °C results were obtained from reactions with the specific anti Ig and anti C_3/C_4 sera using a microtiter plate technique

RESULTS

Characterization of Anti Co^b

As shown in Table 1 anti Co^b consist of IgG without ability of fixing complement In accordance with this the anti Co^b is *in vitro* unable to agglutinate erythrocytes suspended in saline do not show hemolysis and inactivation of serum by heating to 56 °C in 30 minutes does not weaken the reaction between the sensitized cells and the broad spectrum antiglobulin reagent used for AHG

Development of the Co^b Antigen at Birth

Samples from 186 mothers and their 188 newborn babies (including two pairs of twins) were investigated for the presence of the Co^b antigen Table 2 shows that half of the children (7 out of 13) of heterozygous Co(a + b +) mothers inherit the Co^b gene which is expressed at birth and that the number of Co(b +) children equals the number of Co(b +) mothers among the investigated This would be expected for a Mendelian characteristic with full penetrance

Figure 1 shows the antigenic strength of the Co^b antigen in the persons found to be Co(b +) The strength as determined by titration is in every instance compared to the same simultaneously titrated control sample and the deviation in titer from this control is shown in the diagram Measured in this way the strength of the Co^b antigen appears remarkably constant and no difference between children and adults could be demonstrated

TABLE 2 *Colton Phenotypes of 186 Mothers their 188 Newborn Children and 86 Blood Donors*

Mothers	Newborns Co(b–)	Co(a + b +)	Co(a–b +)	Mothers in all	Donors investigated	Adults in all
Co(b–)	166 (168)[a]	6	0	172	79	251
Co(a + b +)	6	7	0	13	7	20
Co(a–b +)	0	1	0	1	0	1
	174 +	14	0	186	86	272

[a] including two pairs of twins

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IN VITRO STIMULATION OF BLOOD LYMPHOCYTES FROM *MYCOPLASMA PNEUMONIAE* INFECTED PATIENTS WITH PNEUMONIA AND WITH DISORDERS OF THE CENTRAL NERVOUS SYSTEM

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Mogensen H H & Lind K *In vitro* stimulation of blood lymphocytes from *Mycoplasma pneumoniae* infected patients with pneumonia and with disorders of the central nervous system Acta path microbiol scand Sect. C 88 61–65 1980

In 23 patients with *Mycoplasma pneumoniae* (MP) infection (13 with pneumonia and 10 with an acute febrile non bacterial disorder of the central nervous system (CNS)) and in 26 healthy control persons thymidine incorporation of blood lymphocytes stimulated *in vitro* by *killed MP* was studied The lymphocyte response to MP was significantly higher in the pneumonia patients than in the controls In the patients with an acute disorder of the CNS lymphocyte responses to MP tended to be low or normal in lack of pleocytosis in the spinal fluid but were predominantly high when either pleocytosis or a pulmonary infiltrate was present Lymphocyte responses to the mitogens PHA PWM and Con A were normal in all groups The lack of increased responses to MP *antigen in* some of the neurological patients despite a current MP infection may reflect an antigen specific depression or a lack of specific sensitization of their lymphocytes

Key words Lymphocytes *Mycoplasma pneumoniae* central nervous system pneumonia

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The nature of immunity in *Mycoplasma pneumoniae* (MP) infection in man is largely unknown The level of antibodies to MP in blood is partly correlated with resistance to reinfection (13) The presence of specific antibodies in bronchial secretions has been demonstrated after infection with MP (4 5 8) [illegible] these secr[illegible] resistance [illegible] volunteers [illegible] MP can survive for a long period in the [illegible] such [illegible] with [illegible] was [illegible] of the infection to the respiratory tract (6) However, the significance of cell mediated immunity in the human MP infection is not clear

Increased responsiveness of lymphocytes to MP antigen after MP pneumonia in man has been [illegible] central nervous system (CNS) disorder and positive tests for complement fixing (CF) antibodies to MP

The aim of the present study was to compare the lymphocyte responses to MP in persons with current or previous MP pneumonia and in control persons and to examine and compare the response

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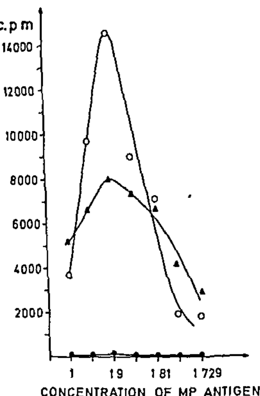


Fig 1 ^{14}C thymidine uptake by lymphocytes after stimulation with varying doses of killed *Mycoplasma pneumoniae* (MP) antigen The lymphocytes were obtained from an MP infected patient (PJ Table 2) on three occasions ● 3 3 77 ▲ 17 3 77 and ○ 18 4 77 Onset of disease 14 2 77 MP antigen dilution 1 is equal to the stock solution

on six occasions within the first year after onset of illness lymphocyte responsiveness to MP was significantly lower than in the controls ($p < 0.01$) Mean of responses was 906 c p m , range 256–1882 c p m For the controls the mean was 4663 c p m , range 560–8422 Lymphocyte responses to PHA, PWM and Con-A were normal to high in this patient

c) CNS disorder The lymphocyte responses to killed MP in patients with meningitis, encephalitis acuta meningo-encephalitis acuta or polyradiculitis acuta plus a positive anti MP CF test are given in Table 2 by a ratio based on the relation to the mean of lymphocyte responses in the controls (see MATERIAL AND METHODS) The patients were in most cases examined on various occasions since lymphocyte responsiveness is often depressed during the first time of infectious disease or later during complicating events, the highest responses obtained within the first 10 months of investigation are therefore considered relevant to an evaluation of the specific sensitization of the lymphocytes It is seen that in patients without a pulmonary infiltrate lymphocyte responsiveness tended to be low or normal in lack of pleocytosis in the CSF, but high in two out of three patients where pleocytosis was present (in the third patient, JM there was only one early examination) When a pulmonary infiltrate was present lymphocyte responsiveness was markedly increased, regardless of the number of cells in the CSF Briefly, lymphocyte responsiveness tended to be low or normal in lack of a focus with cellular infiltration (lungs or CNS) and high in the presence of such a focus

Mitogens

In order to compare the results of non-specifically stimulated lymphocytes, the thymidine incorporation was measured after mitogenic stimulation with PHA PWM and Con A Table 3 shows the results for the MP infected persons within 6 weeks after onset of illness and for the controls Only the nine

TABLE 1 *The Maximal Response to Killed* Mycoplasma pneumoniae *(MP) of Lymphocytes Obtained from 13 Patients with Current or Previous MP Pneumonia in Various Periods after Onset of Pneumonia and from 26 Healthy Control Persons*

	Years after disease	Number of persons tested	Number of observ ations	Range of responses (c p m)	Mean of responses (c p m)
MP pneumonia (normal course)	0–1	9	26	3285–24294	11605
	1–2	8	8	884– 9941	6661
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	3–4	2	2	6132– 7774	6953
	4–5	2	2	5367– 8130	6749
Controls		26	26	560– 8422	4663

to MP *in vitro* of lymphocytes from patients with various neurological manifestations plus serological evidence of current or recent infection with MP

MATERIAL AND METHODS

Subjects Six males and seven females aged 1–64 years (average 28 years) with current or previous MP pneumonia were studied In nine of the patients the study was initiated within 3 months after onset of disease and they were all reinvestigated at least once within the first year (a total of 26 observations) Seven of these patients were further investigated on one or more occasions up to 3 years after onset of disease Four patients who were included in a previous report (12) were each examined on two occasions between 1 and 5 years after onset of illness The diagnosis was in all cases established by a combination of pneumonia (in 10 cases confirmed by X ray in three cases by pulmonary auscultation only) and a positive CF test for antibodies to MP The clinical course was normal with a febrile period of less than 2 weeks Furthermore a 27 year old man with a long lasting and severe MP pneumonia was investigated He was previously healthy without propensity for unusual courses of infectious diseases For almost 3 months he was febrile (38–39 5 °C) periodically with prominent cough and dyspnoea Bilateral pulmonary infiltrations were demonstrated by X ray CF antibody titre was maximally 512 tests for cold agglutinins were negative

Four males and six females aged 5–57 years (average 25 years) with a CNS disorder plus a positive anti MP CF test were examined on various occasions within 10 months after onset of disease altogether 23 times These persons are further described in Table 2 Four of them were also included in the previous report (12) Pleocytosis is defined as the presence of more than 10 leucocytes/µl in the spinal fluid (CFS)

Twenty six healthy volunteers aged 3–49 years (average 26 years nine males and 17 females) without known experience of MP infection served as controls They were all negative when tested for CF antibodies to MP and for cold agglutinins

Lymphocyte cultures The lymphocyte transformation technique has been described in detail previously (11) Briefly mononuclear cells were isolated on a Ficoll Isopaque gradient washed three times and resuspended in RPMI 1640 containing 15 per cent pooled serum (negative for CF antibodies to MP) from healthy young non transfused male donors According to the results of cell titration experiments (see below) 10^5 cells were cultured per vial (round bottomed) in 500 µl medium at 37 5 °C in 5 per cent CO_2 Cultures were harvested after 72 hours when stimulated with mitogens and after 120 hours when stimulated with MP antigen (see RESULTS) ^{14}C thymidine was added 24 hours before termination and the incorporation was quantitated by liquid scintillation counting The results given as counts per minute (c p m) are means of triplicate determinations after subtraction of the values obtained in unstimulated cultures A response in a stimulated culture is considered positive when it is more than 2 5 times the value of the corresponding unstimulated culture Four of the patients in Table 2 have been described in the previous study (12) Because lymphocyte responses were consistently lower in that study than in the present, a ratio (response in patient/mean of the responses in the corresponding controls) was calculated for each study

Antigen preparation The Mac strain of MP was grown in standard mycoplasma medium on the inside of Roux flasks washed scraped off and washed again (10) After dilution 1 5 in phosphate buffered saline pH 7 2 the preparation was ultrasonicated three times 1 minute and stored frozen in small aliquots This non viable preparation had a protein concentration of 0 5 g/litre In all experiments a dose titration was carried out Seven 3 fold dilutions were used added by 20 µl to the cultures The maximal response is defined as the highest value obtained by any of the varying antigen doses The mycoplasma medium (before and after incubation at 37 °C for 5 days) was tested as a control

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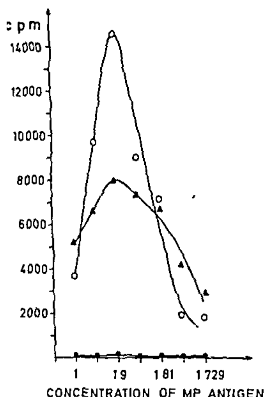


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a) MP pneumonia (normal course) The maximal response to killed MP in these 13 patients on various occasions is compared with the controls in Table 1 The responses obtained in nine patients within the first year after pneumonia were significantly higher than in the controls ($p < 0.01$) so were the responses obtained during the periods 1–5 years and 2–5 years after illness ($p < 0.05$)

b) MP pneumonia (severe course one patient) The person with severe pneumonia was examined

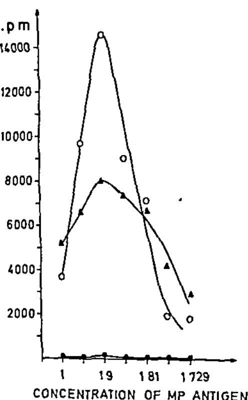


Fig 1 ^{14}C thymidine uptake by lymphocytes after stimulation with varying doses of killed *Mycoplasma* [illegible] Onset of disease 14 2 77 MP antigen dilution 1 is equal to the stock solution

on six occasions within the first year after onset of illness lymphocyte responsiveness to MP was significantly lower than in the controls ($p < 0.01$) Mean of responses was 906 c p m , range 256–1882 c p m For the controls the mean was 4663 c p m , range 560–8422 Lymphocyte responses to PHA, PWM and Con A were normal to high in this patient

c) CNS disorder The lymphocyte responses to killed MP in patients with meningitis, encephalitis acuta, meningo-encephalitis acuta or polyradiculitis acuta plus a positive anti MP CF test are given in Table 2 by a ratio based on the relation to the mean of lymphocyte responses in the controls (see MATERIAL AND METHODS) The patients were in most cases examined on various occasions, since lymphocyte responsiveness is often depressed during the first time of infectious disease or later during complicating events, the highest responses obtained within the first 10 months of investigation are therefore considered relevant to an evaluation of the specific sensitization of the lymphocytes It is seen that in patients without a pulmonary infiltrate lymphocyte responsiveness tended to be low or normal in lack of pleocytosis in the CSF, but high in two out of three patients where pleocytosis was present (in the third patient, JM, there was only one early examination) When a pulmonary infiltrate was present, lymphocyte responsiveness was mar- [illegible] of such a focus

Mitogens

In order to compare the results of non-specifically stimulated lymphocytes the thymidine incorporation was measured after mitogenic stimulation with PHA PWM and Con A Table 3 shows the results for the MP infected persons within 6 weeks after onset of illness and for the controls Only the nine

TABLE 1 *The Maximal Response to Killed* Mycoplasma pneumoniae *(MP) of Lymphocytes Obtained from 13 Patients with Current or Previous MP Pneumonia in Various Periods after Onset of Pneumonia and from 26 Healthy Control Persons*

	Years after disease	Number of persons tested	Number of observ ations	Range of responses (c p m)	Mean of responses (c p m)
MP pneumonia (normal course)	0–1	9	26	3285–24294	11605
	1–2	8	8	884– 9941	6661
	2–3	4	4	7920– 9358	8635
	3–4	2	2	6132– 7774	6953
	4–5	2	2	5367– 8130	6749
Controls		26	26	560– 8422	4663

TABLE 2 *Lymphocyte Responses in vitro to Mycoplasma pneumoniae (MP) Antigen in 10 Patients with an Acute Disorder of the Central Nervous System Given by the Ratio Highest Response in Patient within the First 10 Months/Mean of Responses in Controls*

Patient	Sex	Age (years)	Clinical diagnosis	Anti-MP CF titre		Pulmonary infiltrates	Cells/μl in CSF	Patient/control ratio
				maximum	change			
FTJ[a]	M	23	E	128	Fall	–	0	0.9
HRM	F	22	PR	64	Rise/fall	–	2	1.2
KMH	F	29	E	1000	Fall	–	3	0.7
SS[b]	M	11	E	64	–	–	10	0.6
JM	F	57	ME	256	–	–	89	0.3
JH	M	21	M	2000	Fall	–	190	3.5
PJ	F	21	ME	256	Rise/fall	–	256	3.1
AH	F	10	PR	1000	Rise/fall	+	3	1.9
GS	M	5	E	128	Rise/fall	+	10	4.4
BLH	F	51	M	1000	Rise	+	45	2.7

Clinical diagnoses Encephalitis acuta (E) meningitis (M) meningoencephalitis acuta (ME), polyradiculitis acuta (PR) Additional diagnoses [a]Respiratory syncytial virus infection (rise in antibody titre 8 to 64) [b]Reyes' syndrome Complement fixing (CF) antibodies to MP, presence or absence of pulmonary infiltrates and maximal number of leucocytes per μl in spinal fluid (CSF) are indicated

TABLE 3 *Lymphocyte Responses to Optimal Concentrations of PHA PWM and Con-A in 15 Patients with* Mycoplasma pneumoniae *(MP) Infection (9 Patients with Pneumonia and 6 with an Acute Disorder of the Central Nervous System (CNS)) and in 26 Healthy Control Persons*

		PHA	PWM	Con A
MP pneumonia (n = 9)	Mean c p m	16140	3131	11199
	(SD)	(5507)	(1511)	(5175)
CNS disorder (n = 6)	Mean c p m	22630	3993	14972
	(SD)	(8168)	(938)	(7806)
Controls (n = 26)	Mean c p m	21280	4797	13713
	(SD)	(7796)	(1440)	(5013)

patients with MP pneumonia and the six patients with a CNS disorder participating in the last part of this study (see MATERIAL AND METHODS) were included The differences between patients and controls were not statistically significant, except for the responses to PWM in the pneumonia group ($p < 0.05$)

DISCUSSION

In the present study, blood lymphocytes from individuals with a current or previous MP pneumonia showed increased thymidine incorporation when stimulated with MP antigen as compared to the controls This is in agreement with the results reported by others (2, 7, 9, 12) Although significantly weaker than in these patients, lymphocytes from control persons did respond to MP antigen It is likely that most of the controls had had an MP infection in the past, although no serological evidence hereof could be obtained (2, 7) Investigations by *Biberfeld* (1) indicate that the proliferative response *in vitro* of human lymphocytes to MP is an antigen-specific T-cell response rather than a non-

antigen-specific mitogenic effect as demonstrated for mouse and guinea pig spleen lymphocytes (3) Besides it cannot be excluded that MP possesses antigens in common with other microorganisms giving unspecific cross reactions

In one patient with an unusual severe and protracted MP pneumonia lymphocyte responses to MP were even lower than in the controls No observations were made that could elucidate the connection between the clinical course and the low specific lymphocyte response in this patient

In four of the patients with serological evidence of an MP infection and a CNS disorder who had neither pleocytosis nor pulmonary infiltrates a low or normal lymphocyte response to MP was found while increased responses were seen in five out of six patients where such cellular accumulations had been present This could suggest a positive relation between the blast transformation of lymphocytes *in vitro* and the cellular accumulation *in vivo*

The normal responses of lymphocytes to unspecific stimulation with mitogens in both groups of MP infected patients indicate that the lack of an increased response to MP antigen in some of the neurological patients may reflect either an antigen specific depression or a lack of specific sensitization of their lymphocytes

Since reactions of circulating lymphocytes may not give proper information concerning cellular reactions in the lung a common site of MP infection in man and since lymphocytes from this site are not accessible studies have been performed on lymphocytes from tonsils and adenoids another site of MP infection (to be published)

Mrs *Inge Torp* is thanked for excellent technical assistance The permittance to study patients admitted to the Pediatric Department Centralsygehuset Holbæk and to the Department of Pulmonary Disease Frederiksberg Hospital is appreciated This study was supported by the *Danish Medical Research Council* (grants no 512-7215 and 518-8772)

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FRAGMENTS AND SUBCLASSES OF IgG IN ELUATES OF HUMAN MALIGNANT TISSUES

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Wesenberg F Fragments and subclasses of IgG in eluates of human malignant tissues Acta path microbiol scand Sect C 88 67–71 1980

Eluates of 13 malignant tumours were prepared at 56 °C using the continuous flow technique By using immunodiffusion techniques 50–80 per cent of the IgG detected was found to be of the IgG1 subclass The ratio of Ig/kappa to Ig/lambda was similar in eluates and in the corresponding extracts and this ratio was similar to that obtained using pooled human serum This indicates a normal distribution of IgG subclasses in the eluates Besides whole IgG the eluates and corresponding extracts contained fragments of IgG This was revealed by using sodium dodecyl sulphate electrophoresis (SDS PAGE) However since parts of the IgG associated with human malignant tumours can be non specifically bound and since fragments of IgG was found in extracts and eluates of normal tissues although to a lesser degree than in those of malignant tissue no conclusive evidence was obtained that the malignant tissue could degrade Ig SDS PAGE of extracts and eluates of malignant tissues showed 2–3 constant bands not detected in isolated IgG or in extracts and eluates of most of the normal tissues These bands were not identified

Key words Human tumours IgG subclasses IgG fragments

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Eluates of human malignant tissues contain both immunoglobulins (reviewed in 15) and other proteins (8, 11), but few reports have dealt with IgG

fragments in eluates of human leukemic blast cells indicating that the IgG was degraded on the cells Further Wu et al (16) have shown that extracts of human malignant tissue could degrade human IgG at low pH

We have previously shown that eluates of human malignant tissue contain IgG (13) and antiproteases (11) This study was designed to investigate which fragments of IgG and IgG subclasses were present in these eluates

MATERIALS AND METHODS

Extracts and Eluates

al tissues were the same as used previously (11 13) having been stored at −25 °C In brief they had been prepared in the following way The tissue had been obtained at autopsies and had been minced with scissors homogenized for 1 min in a Servall Omni Mixer and centrifuged at 20 000 × g for 20 min The supernatant was called the extract. The sediment was placed between glass fibre filters in a short glass column and was washed in a continuous upward flow of phosphate buffered saline pH 7 2 (PBS) with 0 05 per cent Na azide at 4 °C The column was then submerged in a waterbath and the tissue eluted in the continuous flow of PBS at 37 45 and 56 °C successively The eluates were

TABLE 1 *IgG1 Eluted from Human Malignant and Normal Tissue in Per Cent of Total IgG*

Type of tissue	No	Mean per cent IgG (range)
Malignant	13	63 (50–80)
Normal	8	68 (60–80)

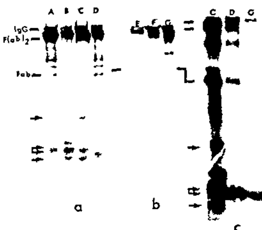


Fig 1 Sodium dodecyl sulphate electrophoresis of precipitates made by mixing antiserum to human gamma kappa and lambda chains with eluates of
A Carcinoma of the bronchus
B Fibrosarcoma
C Carcinoma of the cervix
D Carcinoma of the breast
E Normal muscle
F Normal lung
G Normal kidney
12 5 per cent polyacrylamide gel used in the separating gel The large band found between IgG and F(ab)$_2$ using stained gels are the rabbit IgG used to produce the precipitates

Single arrow	Bands only present in eluates of malignant tissue
Double arrow	Band present in all eluates of malignant tissue and some eluates of kidney from older individuals
a and b	stained gels
c	autoradiography

(aged 50–60 years) and in eluates of normal lung stomach and muscle from individuals of various ages (20–60 years) The IgG1 amounted to 60–80 per cent of the total IgG (Table 1)

Ig/kappa and Ig/lambda

The ratio of concentration of Ig/kappa and Ig/lambda to the concentration of IgG was similar in eluates and corresponding extracts and corresponded to the results obtained using PHS

Fragments of IgG

All the eluates of malignant tissues gave precipitation lines against antisera to IgG/Fab IgG/Fd and IgG/Fc using the double diffusion test in agar In order to further characterize the eluates they were mixed with the antiserum to human gamma kappa and lambda chains and the proteins precipitated were separated using SDS PAGE To check the specificity of the antiserum isolated IgG pepsin and papain digested IgG were similarly mixed with the antiserum The three preparations were precipitated by the antiserum SDS PAGE of the antiserum showed only one line indicating that the antiserum used contained no fragments of rabbit Ig This band appeared between the IgG and the F(ab)$_2$ band and was rather large However the amount antisera used were found optimal for precipitating the fragments used for control

Representative results are shown in Fig 1a All the eluates showed lines corresponding to whole IgG and IgG/Fab Using stained gels it was difficult to separate the smaller IgG/ F(ab)$_2$ band from the larger rabbit Ig band only in B this band was clearly seen Therefore autoradiography was performed with ^{125}I labelled eluates The F(ab)$_2$ band was now clearly demonstrated in the other eluates (Fig 1c) Several other bands appeared However using stained gels 2–3 bands appeared only in the eluates of the malignant tissues (arrows Fig 1) and one of these appeared in all the eluates

Extracts of the tissues gave similar results to that obtained using the correspon ding eluates

Using eluates of the norm[illegible]

[illegible] eluates of normal muscle gave precipitation lines against IgG/Fab and IgG/Fc Representative results using SDS PAGE are shown in Fig 1b All the eluates showed lines corresponding to whole IgG and eluates of normal muscle (E) gave only this line Eluates of kidney from older individuals (G) and eluates of normal lung (F) and stomach (not shown) gave several other lines two of them corresponded to IgG/F(ab)$_2$ and IgG/Fab (Fig 1b + 1c) However as mentioned above none of the eluates of normal organs gave the

concentrated to between 1 5 and 2 5 ml using Amicon Diaflo UM 05 filters The concentration of IgG in extracts and eluates was determined using single radial immunodiffusion technique The immunodiffusion plates were purchased from Behringwerke, Marburg-Lahn, West Germany For the experiments here described, only the extracts and the 56 °C eluates were used

Sera

Pooled human serum (PHS) was obtained by mixing serum from at least 20 healthy blood donors Isolated IgG was purchased from AB Kabi, Stockholm Rabbit antisera to human Ig/kappa, Ig/lambda, IgG/Fab IgG/Fd and IgG/Fc were purchased from Behringwerke, Marburg-Lahn West Germany A rabbit antiserum with specificity to human gamma, lambda and kappa chains was purchased from DAKO immunoglobulins, Copenhagen Swine antisera to subclasses of human IgG1–4 were purchased from Nordic Immunological Laboratories Tilburg The Netherlands The specificity of the antisera was confirmed by double diffusion test in agar using mixtures of antigens and isolated antigen preparations

Double Diffusion Test

The double diffusion test for IgG subclasses was performed according to the instructions recommended by the manufacturer of the antisera Otherwise, the test was performed as described previously (12)

Quantification of IgG Subclasses

A single radial immunodiffusion technique was used Antisera to the various subclasses were mixed with 2 0 ml 1 per cent agar at the optimal condition for quantification and poured onto glass plates 76 × 26 mm Holes of 3 mm in diameter were punched out Ten or 20 µl of eluates were applied PHS at varying dilutions were used as standards The IgG content of the PHS was 10 5 g/l Assuming that the distribution was normal (7) the content of IgG1 was calculated to be 6 5 g/l IgG2 3 0 g/l IgG3 0 6 g/l and IgG4 0 4 g/l The glass plates were left in a moist chamber at circa 20 °C At 48 h the plates were washed and stained using Coomassie blue The standard curve was drawn and the content of the subclasses found The amount of the subclasses were related to the total amount of IgG and the per cent of the subclasses calculated

Ig/kappa and Ig/lambda

Quantification of the total amount of Ig with kappa and Ig with lambda chains in extracts and eluates was performed as described for IgG subclasses using antisera to Ig/kappa or Ig/lambda in the agar Isolated IgG was used as the standard and 65 per cent was assumed to be IgG with kappa chain and 35 per cent IgG with lambda chain (7) The amount found was related to the total amount of IgG and the ratio of concentration of Ig/kappa or Ig/lambda to the concentration of IgG in eluates was compared to the ratio found using the corresponding extracts

^{125}I labelling of IgG and Eluates

Human IgG and some of the eluates were labelled with ^{125}I using the chloramin-T method described by *Hunter* (4) 100 µl of eluates were labelled with 0 25 mCi ^{125}I (Institutt for Atomenergi, Kjeller, Norway) 100 µg of human IgG was labelled with 1 mCi ^{125}I

Sodium Dodecyl Sulphate Electrophoresis (SDS-PAGE)

100 µl of eluates or suitable dilutions of extracts were mixed with 50 µl of the antiserum to human gamma kappa and lambda chains The mixtures were incubated for 30 min at 37 °C followed by 18 h at 4 °C The precipitates obtained were washed twice in PBS and once in distilled water They were then resuspended in a 0 125 M Tris HCl buffer, pH 6 8 with 2 per cent SDS 10 per cent glycerol and 0 001 per cent bromphenol blue and incubated for 18 h at 37 °C followed by incubation at 100 °C for 2 min The proteins were separated using SDS-PAGE in slab gels following in detail the method described by *Laemmli & Favre* (5) using 6 0 per cent polyacrylamide in the »stacking« gel and 12 5 per cent polyacrylamide in the »separating« gel The separated proteins were fixed in trichloroacetic acid and stained with Coomassie blue Isolated IgG pepsin digested IgG papain digested IgG and human serum albumin were [illegible]

& Avsonoff (9)

Autoradiography

^{125}I labelled eluates were precipitated with non labelled antiserum and the proteins separated using SDS PAGE as described above After staining, the gel was dried (Gel slab dryer, model 224 BIO RAD laboratories) layered on Kodak XS 1 film and after exposure developed in a Kodak R P X Omat Processor

RESULTS

IgG Subclasses

Using the double diffusion test, only IgG1 was detected in some of the eluates The single radial immunodiffusion test was thereafter performed Different amounts of antisera were used in the gel for optimal quantification conditions Differing dilutions of PHS were used as standards IgG1 was detected in all eluates of malignant tissue and amounted to 50–80 per cent of the total IgG (Table 1) This means that most of the IgG eluted from the tissue was IgG1 IgG2–4 were not detected in any of the eluates This may well be due to the low concentration of IgG, since when using PHS as standard more than 5 times the highest concentration of total IgG in the eluates had to be used to detect these subclasses in PHS

IgG1 was also the only subclass detected in eluates of normal kidney from older individuals

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lines indicated with the arrows in Fig 1a using stained gels Autoradiography showed however that some of the eluates of the kidney from older individuals also contained the band indicated with the double arrow (Fig 1c) Extracts of the tissues gave results similar to those obtained using the corresponding eluates

To investigate whether the IgG was degraded during the elution procedure ^{125}I labelled human IgG was mixed with extracts of malignant tissue The mixture was kept at 4 37 45 and 56 °C parallel to the procedure used for preparation of the eluates (13) The proteins of the mixture were separated using SDS PAGE No fragments of the ^{125}I labelled IgG were found

Eluates of kidney from younger individuals (aged 20–30 years) and eluates of liver and spleen from individuals of various ages did not precipitate any of the antisera mentioned above

DISCUSSION

The results presented indicate that there seems to be a normal distribution of the IgG subclasses in eluates of human malignant and normal tissues since 50–80 per cent of the eluted IgG was of the IgG1 subclass there was the same relationship between Ig/kappa and Ig/lambda in the eluates and corresponding extracts and the results corresponded to those obtained using PHS However compared to PHS the IgG concentration had to be raised more than 5 times to detect IgG2–4 in the eluates This means that there can be an increase in one of those subclasses but too small to be detected *Romsdahl & Cox* (8) detected IgG4 in the eluates of human sarcomas using electrofocusing techniques but could not quantify the amount Consequently they could not show if the concentration of this subclass was increased compared to the other IgG subclasses *Daveau et al* (3) showed that the concentration of IgG4 was increased in sera from patients with advanced melanoma but it is difficult to conclude that this finding is representative of the tumour associated Ig There seems therefore to be no evidence yet given that there is not a normal distribution of IgG subclasses in eluates of human malignant tissues

Another question raised has been if the tumour associated Ig is degraded (15) The results obtained here show that besides whole IgG the extracts of human solid tumours contained F(ab')$_2$ and Fab fragments of IgG This could mean that the degradation of human Ig obtained at low pH *in vitro* using extracts of human malignant tissue (16) also could occur *in vivo* But since degraded IgG was found in extracts of normal organs the results could reflect the normal degradation that occurs in tissues

Of more interest are the findings that in addition to whole IgG fragments of IgG were found in all eluates of malignant tissue This could mean that the IgG attached to the malignant cell was degraded *in vivo* as indicated by *Cotropia* (2) who detected Fab fragments on human leukemia blast cell and by *Romsdahl & Cox* (8) who found free Fab fragments in eluates of human sarcomas The finding of antiprotease in eluates of malignant tissues (8 11) could also support this interpretation as discussed by *Romsdahl & Cox* (8) On the other hand we have previously shown that part of the tumour associated IgG is non specifically bound (11 13) Furthermore the results obtained here show that eluates from some normal organs especially normal kidney from older individuals have detectable amounts of fragments of IgG The IgG associated with these organs is non specifically bound (11 13) Further investigations are therefore necessary to clarify if the tumour associated Ig are degraded on the cell

The method used did not allow quantification of the amount of IgG fragments but apparently much of the IgG was not degraded (Fig 1) This is important for quantification of IgG and IgG subclasses since the antisera used require an intact IgG molecule The experiments using ^{125}I labelled human IgG showed that the degradation of IgG in the eluates must have occurred *in vivo* since no degradation occurred *in vitro* during the elution procedure

SDS PAGE of extracts and eluates of malignant tissue showed 2–3 constant bands that were also detected in some eluates of the kidney from older individuals but not detected in extracts and eluates of the other normal tissue in isolated IgG or in pepsin or papain digested IgG One of the bands found in the eluates of malignant tissue could be the Fcγ receptors (FcR) eluted either from the tissue or eluted with the IgG These FcR have been claimed to have a molecular weight ranging from 18000 to 130000 daltons (1 6) The 2–3 bands may also be tumour specific or other antigens eluted from the tissue and which have recombined with the specific antibody These results indicate that eluted antibodies also can be found as antigen-antibody complexes in the eluates

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ACTIVATION OF COMPLEMENT BY ANTIBODIES TO THE KERATOSULPHATE-LIKE HOST ANTIGEN OF SENDAI VIRUS

PER FADNES and GUNNAR HAUKENES

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Fadnes P & Haukenes G Activation of complement by antibodies to the keratosulphate like host antigen of Sendai virus Acta path microbiol scand Sect C 88 73–76 1980

Sendai virus is haemolytic against erythrocytes from different species Pre treatment of Sendai virus with antibody and complement (C) enhances the haemolytic activity of the virus This property was used to examine the pathway by which C is activated by Sendai virus antigen antibody complexes Antibody against the keratosulphate like host antigen was used in a monospecific antigen antibody system Evidence is presented that both the classical and the alternative pathway is activated the alternative to a much lower extent

Key words Sendai virus complement alternative pathway classical pathway

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All studies so far concerning the complement (C) activating mechanism of virus antigen antibody complexes have been performed with virus infected cells

Human IgG and F(ab')$_2$ have been shown to cause lysis of measles virus infected cells by activation of the alternative C pathway (9 10) *Ehrnst* (2) has shown that the alternative C pathway is activated by antibodies to the haemagglutinin antigen and that the classical pathway is activated by antibodies to the haemolysin antigen of measles [illegible]

[illegible] ...mediated increase in haemolysis was shown to be a result of an indirect haemolysis (3)

In the present study we have examined the pathway by which antibody to the keratosulphate like host antigen of Sendai virus activates C

MATERIALS AND METHODS

Virus

Parainfluenza I virus (Sendai) was propagated in the chick allantoic cavity at 35 °C for 2 to 3 days The virus was quantitated by haemagglutination (HA) [illegible]

Antisera

Antiserum against the keratosulphate like host antigen was produced by immunization of rabbits with allantoically grown [illegible]

[illegible] ...g for 1 h before being suspended to give 4 HA units Addition of purified host antigen to the antiserum blocked the HI activity

Complement (C)

Serum from normal guinea pigs (ngpC) or guinea pigs deficient in the fourth component of complement (C4 def gpC) were used as complement source The sera were stored at −70 °C

Buffer

Studies of C activity were performed using a barbital buffer of pH 7 3 made isotonic by 0 8 per cent NaCl and supplemented with 0 15 mM $CaCl_2$ and 0 5 mM $MgCl_2$ (C buffer) To stop the activation of C 10 mM og ethylene diamine tetraacetate (EDTA) was added

Preparation of IgG and F(ab)₂

IgG from the rabbit antiserum against the host antigen was isolated from a DEAE cellulose column by elution with 0 0125 M phosphate buffer pH 6 9 and thereafter concentrated by ultrafiltration The purified IgG gave only one precipitation line on immunoelectrophoresis against antiserum to whole rabbit serum For preparation of F(ab $)_2$ the purified IgG preparation was digested by pepsin as described by *Reid* (11) The F(ab $)_2$ was fractionated and isolated by gel filtration through Sephadex G 150 A single peak with a K_{av} of 0 19 typical of F(ab $)_2$ was obtained The separated F(ab $)_2$ showed no precipitation line against an anti Fc antiserum The test system enabled the detection of ≥ 0 03 g IgG per l

Sendai Virus Haemolysis (SVH)

Sendai virus was pre treated by heat inactivated (56 °C 30 min) antiserum or antibody preparation and C in glass tubes at a total reaction volume of 0 3 ml consisting of 0 1 ml of Sendai virus in allantoic fluid (HA titre 1 024) 0 1 ml of antibody dilution and 0 1 ml of C dilutions Controls in which either antibody or C was replaced by C buffer were always included

The reaction mixture was incubated at 37 °C for 1 h Thereafter 2 ml of a 2 per cent suspension of chick erythrocytes in barbital buffer with 10 mM EDTA and without Ca^{++} and Mg^{++} were added The tubes were further incubated for 1 h at 37 °C and repeatedly shaken to prevent sedimentation of the erythrocytes The erythrocytes were then pelleted at 3 600 × *g* for 5 min and the optical density (OD) of the supernatant was read in a Hitachi spectrophotometer at 540 nm

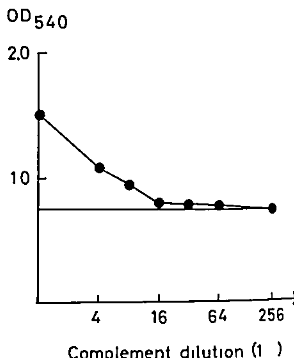


Fig 1 Haemolytic activity (OD_{540}) of Sendai virus before and after addition of normal C
●—● C added
——— Untreated virus

RESULTS

Haemolytic Activity of Sendai Virus Antibody Complexes Treated with Normal and C 4 def C

Normal gpC always caused an increase in SVH at low dilutions without the addition of antiserum (Fig 1) This haemolysis was always much lower than that obtained when antiserum was added and it was rapidly lost when the C was further diluted Attempts to find HI antibodies against Sendai virus in the gp serum were not successful A similar effect was never observed with C 4 def gpC

Using antiserum to the keratosulphate like host antigen diluted 1 16 both normal and C 4 def gpC showed a significant increase in SVH (Fig 2) Normal gpC showed a higher activity than C 4 def gpC

The C had to be kept within a certain range of dilutions to obtain maximal haemolysis which indicated that the C serum together with antibody inhibit the contact between virus and erythrocytes

Using IgG separated on DEAE-cellulose chromatography as the antibody source the C mediated increase in SVH with ngpC was the same as that obtained when antiserum was used However the increase in haemolysis caused by IgG and C 4 def C was considerably lower (cf Table 1 and Fig 2) This haemolysis also seemed to be dependent on high IgG concentrations The same degree of haemolysis was observed when the virus was first sensitized with antiserum for 16 h at 4 °C and soluble serum factors thereafter removed by pelleting the virus antibody complexes in the ultracentrifuge at 100 000 × *g* for 1 h

C 4 def gpC at any dilution was never haemolytic against sheep erythrocytes optimally sensitized by antibody (EA) Adding heat inactivated rabbit serum at low dilutions rendered the C 4 def gpC haemolytic against EA

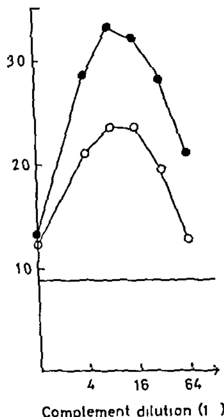


Fig * SVH (OD540) before and after addition of antiserum diluted 1 in 16 and normal or C 4 def C
●—● Normal C
O—O C 4 def C
——— Haemolytic activity of untreated virus

Haemolytic Activity of Sendai Virus – F(ab')2 Antibody Complexes Treated with Normal and C 4 def gpC

The haemolytic activity of Sendai virus was examined using pepsin-digested IgG (F(ab')2) as antibody

TABLE 1 *Increase in Sendai Virus Haemolysis (OD540) after Addition of Purified IgG Antibodies and C 4 def C*[a]

IgG g/l	Guinea pig C 4 def C dilution 1 2	1 4	1 8	1 16
2	0 15	0 30	0 58	0 43
1	0 00	0 08	0 33	0 30

a) Haemolysis with unfractionated antiserum is shown in

TABLE 2 *Increase in Sendai Virus Haemolysis (OD540) After Addition of F(ab')2 and Normal or C 4 def C*

F(ab')2 g/l	Guinea pig C4 def C dilution 1 2	1 4	1 8	Normal guinea pig C dilution 1 2	1 4	1 8
2 0	0 085	0 43	0 67	0 10	0 45	0 65
0 5	0 00	0 15	0 20	0 075	0 18	0 30

Both normal and C 4 def gpC gave a similar and considerable increase in SVH (Table 2) The haemolysis level was the same as that obtained when C 4 def gpC and IgG were used

Relatively high concentration of F(ab')2 seemed to be necessary for maximal haemolysis whereas high concentrations of C had an inhibitory effect

DISCUSSION

The keratosulphate like host antigen has mostly been studied in influenza virus (4 5 6 7 8) The antigenic determinant group is L fucose bound to D galactose through an α glykosidic linkage (7 8) The same antigenic determinant has been found in both the haemagglutinin and the neuraminidase spikes (4) Antibodies to this antigen show high HI activity with orthomyxoviruses but considerably lower with parainfluenza virus (3) In an earlier study we have demonstrated typical complement mediated holes in the membrane of Sendai virus after treatment with antibody to the host antigen and C (6) The serum in our experiments showed an HI titre against Sendai virus of 16 The specificity of this HI activity was verified by the blocking of the HI activity by purified host antigen Allantoically grown influenza virus and Sendai virus share no other antigen demonstrable by the HI test We therefore use a monospecific antigen antibody system with a known antigenic determinant

Normal gpC caused an increase in SVH at low dilutions without the addition of antiserum This haemolysis may be due to small amounts of antibody to a paramyxovirus in the C serum not detectable by HI titration Because of pre treatment of sera with cholera ...

presumed antibody activated the classical C pathway only

Normal C caused a marked increase in SVH in the presence of antibody Antiserum and separated IgG gave the same increase in haemolysis

When C 4 def C was used in the same way the increase in SVH was much higher when antiserum was used compared to that obtained using separated IgG (cf Table 1 and Fig 2) This difference is probably due to a residual of C 4 in the antiserum having resisted the heat inactivation It is known that some C 4 activity may persist after heat inactivation

The increase in SVH after the addition of C 4 def C and separated IgG antibodies was significant but only about 15 per cent of the increase observed when normal C was used This increase in SVH was dependent on relatively high IgG concentrations High concentrations of C 4 def C seemed to inhibit the haemolysis probably by inhibiting the contact between virus and erythrocytes (non specific inhibitors) Most likely C 3 was activated through the alternative pathway in this case

Using F(ab')$_2$ antibody fragments which are known mainly to activate the alternative pathway *(12) the increase in SVH was the same with normal* and C 4 def C The increase in SVH caused by F(ab')$_2$ and C was the same as that obtained by IgG and C 4 def C Relatively high concentrations of F(ab')$_2$ seemed to be necessary while high concentrations of C inhibited SVH

The results clearly show that the alternative C pathway is activated by antibodies to the keratosulphate like antigen of Sendai virus although the activation through the classical pathway proved more efficient Attempts to activate the alternative C *pathway by human and rabbit antibodies to other* Sendai virus antigens have hitherto failed The antigens concerned are the haemagglutinin the neuraminidase and the haemolysin In our experimental set up such studies are complicated by the lack of monospecific sera as antibodies against all three antigens participate in the inhibition of the binding of Sendai virus to erythrocytes

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11 [illegible]

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DEMONSTRATION OF SEPARATE RECEPTORS FOR HUMAN IgA AND IgG IN GROUP A STREPTOCOCCI TYPE 4

Separation of the Solubilized Receptors from Group and Type-Specific Antigens Lipoteichoic Acid and Peptidoglycan

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Schalen C Christensen P Grubb A Samuelsson G & Svensson M L. Demonstration of separate receptors for human IgA and IgG in group A streptococci type 4 Separation of the solubilized receptors from group and type-specific antigens lipoteichoic acid and peptidoglycan Acta path microbiol scand Sect C 88 77 82 1980

The alkaline extract of group A streptococci type 4 was separated by electrophoresis and diffused against 27 normal human sera One of the precipitates appeared with 85% of the sera Addition of purified IgA myeloma protein or sera containing IgA M-components to the extract changed the electrophoretic mobility of the precipitate anodically Purified IgG Fc fragments or sera containing IgG M-component did not affect the mobility of the precipitate It was concluded that this precipitate contained the streptococcal receptor for human IgA A non precipitating IgG Fc receptor with agglutinating capacity for cells coated with human IgG1 but not IgG3 was localized by preparative electrophoresis to the same electrophoretic region as the IgA receptor The mobility of the IgG receptor remained unaltered on addition of IgA myeloma protein permitting a separation of the two receptors by preparative electrophoresis The receptors were distinct from the group specific carbohydrate peptidoglycan and lipoteichoic acid No M antigen or opacity factor were demonstrated in the extract.

Key words Streptococci M protein peptidoglycan group A carbohydrate lipoteichoic acid human IgA human IgG

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Most streptococci belonging to groups A C and G conta n [illegible]

[illegible] oup A streptococci have varying affinity for [illegible]

[illegible] shown that the Fc receptor for human IgG from [illegible] 15 was precipitated by 83% of normal Swedish sera and that it was distinct from the M protein peptidoglycan and group-specific carbohydrate by electrophoresis (2)

The binding of radiolabelled IgA myeloma protein to some group A [illegible] in 1975 [illegible] existence [illegible] different [illegible] strains and that radiolabelled IgG and IgA were bound to the streptococci independently Among the reference strains of group A streptococci tested a type 4 culture showed an outstanding capacity for binding of IgA Later

When *C 4 def C* was used in the same way the increase in SVH was much higher when antiserum *was used compared to that obtained using separated* IgG (cf Table I and Fig 2) This difference is probably due to a residual of C 4 in the antiserum having resisted the heat inactivation It is known *that some C 4 activity may persist after heat* inactivation

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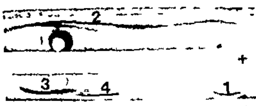


Fig 1 Precipitation pattern on diffusion of electrophoretically separated alkaline extract of type 4 group A streptococci against two different human sera applied in the troughs The precipitates appearing are nominated 1 2 3 and 4 respectively Anode to the right

one serum Precipitates 1 and 2 appeared only in sera containing also precipitate 3 The precipitate 4_{T4} was obtained with two sera which did not give 3_{T4}

Ten µl of one of the sera with precipitates 1_{T4} 3_{T4} and 4_{T4} (serum KP) was added to 10 µl of the type 4 extract and the mixture subjected to electrophoresis On diffusion against 6 sera the precipitates corresponding to 1_{T4} 3_{T4} or 4_{T4} were no longer obtained

Electrophoretic Mobility of the Streptococcal Receptor for IgA of the Alkaline Extract of Type 4 Group A Streptococci

The alkaline extract was mixed with equal volumes of human sera containing high amounts of IgG (8 sera) or IgA (9 sera) M-components After electrophoresis of the mixtures containing IgA M component and diffusion against the normal serum KP 3_{T4} had moved anodally (Fig 2) In contrast 7 of the sera with IgG M-component did not affect the precipitate 3_{T4} while with one IgG M-component serum the precipitate 3_{T4} was no longer visible

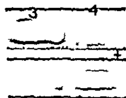


Fig 2 The effect of addition of purified IgA myeloma protein (2 mg/ml) to the alkaline extract of type 4 group A streptococci on the precipitates obtained between the electrophoretically separated extract and serum KP in diffusion experiment Note that precipitate 3_{T4} is shifted anodically while 4_{T4} remains in the same position Troughs normal serum KP lower well application for equal volumes of IgA myeloma protein and alkaline extract upper well application for alkaline extract diluted 1 2 + indicates the anode

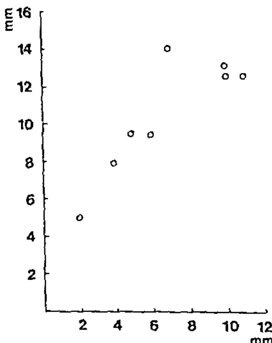


Fig 3 Dislocation of the 3_{T4} precipitate obtained between the type 4 extract and serum KP on addition of human sera containing IgA M-component to the extract set in relation to the electrophoretic mobility of the IgA M-components The position of each precipitate was defined as the point on the arch situated closest to the serum channel Ordinate Distance between the localization of the IgA M-component of each serum and the serum application well in immunoelectrophoresis Abscissa anodal displacement of 3_{T4} by addition of the corresponding serum to the alkaline extract before electrophoresis

The sera with IgA M-component were tested in immunoelectrophoresis with anti IgA serum All M-components were localized anodally in relation to the application well The dislocation of the precipitate 3_{T4} and the electrophoretic charge of the IgA M-components used in the individual experiment correlated ($r = 0\ 82$)

Immunoelectrophoresis of the sera containing IgG M-component revealed that 5 of the M components were situated anodally and 3 cathodally in relation to the application well

Addition of equal volumes of the solution of isolated IgA myeloma protein (2mg/ml) to the extract moved 3_{T4} towards the anode while IgG Fc fragments (0 6 –2 5 mg/ml) did not affect line 3_{T4} or any of the other precipitates

Separation of the Streptococcal Receptor for IgG from the IgA Receptor

After electrophoresis of the extract pieces of gel

group A and G streptococci containing T-type 4 antigen were found to agglutinate in highly diluted sera containing haptoglobin of types 2-1 and 2 2 but not of 1 1 (5, 12)

We now report that group A streptococci possess two separable immunoglobulin receptors one for IgA and one for IgG Furthermore, the relationship of the receptors to some other streptococcal factors was investigated, such as group- and type specific antigens peptidoglycan haptoglobin binding activity and lipoteichoic acid

MATERIALS AND METHODS

Streptococcal Preparations

Alkaline extracts of types 4 (strain SS 241) (alk ex T4) and 15 (strain 100070) (alk ex T15) group A streptococci were prepared from overnight cultures in 20 litres of Todd Hewitt broth as described (2) Several batches prepared on different occasions were studied The protein content of the type 4 extracts as determined by a modified Folin method (8) was 0 5 to 0 9 mg/ml The line 1 components in the alk ex T15 earlier described were isolated by preparative electrophoresis (2) The protein content of this fraction was 1 3 mg/ml

In some experiments alk ex T4 was treated by trypsin as described (2) Peptidoglycan was prepared from type 1 group A streptococci (strain 8198) with hot formamide (13) Purified lipoteichoic acid (LTA) from *S mutans* was kindly supplied by Dr *I Ginsburg* Jerusalem Israel

Human Sera M Components IgG Fc Fragments and Haptoglobin

Sera were collected from 27 apparently healthy individuals among the laboratory staff Seventeen *myeloma sera* 9 containing IgG M component (IgG content 12 to 65 mg/ml) and 8 IgA M component (IgA content 19 to 34 mg/ml) were used The concentration of polyclonal Ig in all sera was below 1 mg/ml The *purified human myeloma proteins* were identical with those previously used (1 2) *Fc fragments* of polyclonal human IgG were prepared as earlier described (1) Three *sera of haptoglobin type* 1 1 3 of type 2 1 and 4 of type 2 2 were randomly selected Haptoglobin typing was performed as described by *Laurell* (7) *Haptoglobin type* 2 2 purified as described by *Laurell* (6) was available at the laboratories

All sera were heated at 56 °C for 30 min and stored at −20 °C until used

Electrophoretic Techniques

Precipitates between electrophoretically separated streptococcal extracts and human sera were done as described previously (2) In brief the streptococcal extract components were separated by electrophoresis and the separated extract then diffused against serum applied in a channel cut in the gel in the direction of the current The precipitates obtained between extract of type 4 group A streptococci and human sera were designated 1_{T4} 2_{T4} etc

Preparative electrophoresis was performed as described (2)

Immunoelectrophoresis of human sera containing M component was performed using rabbit antisera to IgA and IgG (Behringwerke West Germany) 1 2 µl of serum diluted 1 20 was applied and the antisera were used *in 1 5 dilutions*

Rabbit Anti Streptococcal Sera

Difco (Michigan U S A) Anti group A streptococcus serum cross reacting with *S mutans* lipoteichoic acid was kindly supplied by Dr *I Ginsburg*

Test for Streptococcal Opacity Factor (OF)

The presence or absence of OF in streptococcal extract was *investigated by the plate method described by Maxted et al* (9)

Test for Streptococcal Lipoteichoic Acid

The test for the presence of LTA in the streptococcal extract was performed essentially as described by *Ofek et al* (10) *Human group 0 Rh negative red* blood cells suspended to 2% (vol/vol) in saline were treated with 100 µg/ml of papain in the presence of 10 µg/ml of *cysteine for 30 min at 37 °C* The sediment from 0 5 ml of the treated red cells was suspended in 0 5 ml of the fractions to be tested and the mixtures incubated at 37 °C *for 30 min* After three washings in saline a 2% suspension of the cells was again prepared The cells were then tested for agglutination in 0 2 ml volumes of twofold dilutions of anti LTA serum The amount of LTA in the streptococcal extract was given as the agglutination titre obtained with the antiserum The presence of LTA in the extract preparations was further confirmed in inhibition studies using papain treated human red cells coated with purified *S mutans* LTA (10)

Hemagglutination and Hemagglutination Inhibition (HAI) Studies

These tests were performed as described previously using the human anti Rh antibodies Ri KM and HUN (1 14)

RESULTS

After electrophoresis of the extract four main precipitation arches were obtained in diffusion against the sera from 27 apparently healthy individuals (precipitates 1_{T4} 2_{T4} 3_{T4} and 4_{T4}) (Fig 1) Twenty three of 27 sera (85%) gave the 3_{T4} precipitate while 7 of tl

(19%) 4_{T4} *The precipit*

for 3_{T4} resembled the IgG Fc receptor shown in the type 15 extract (2) with respect to electrophoretic mobility and the frequency by which human sera were precipitated However the component from type 4 was apparently different from the type 15 IgG Fc receptor since the latter is shifted anodally [illegible] f IgG Fc fragments [illegible]

anodally in the presence of IgA M-components while IgA M-components did not influence the line 3 of type 15 (unpublished observation) Evidently the type 4 component responsible for line 3_{T4} interacted with IgA independently of the specificity of the antibody combining sites since 9 different IgA M-components caused a shift of the precipitate to the anode This finding indicates that the type 4 component responsible for 3_{T4} represents the earlier described streptococcal receptor for human IgA (4) It is not known whether the IgA receptor is active for both IgA subclasses and secretory IgA

In the fractions corresponding to the 3_{T4} precipitate an *agglutinating capacity for anti Rh* coated human red blood cells was also demonstrated These fractions agglutinated cells coated with IgG1 subclass antibodies but not with IgG3 antibodies The lack of reactivity at the sensitivity [illegible]

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No precipitation was obtained in double diffusion-in gel experiments between the alk ex of T4 and anti type 4 M typing serum No opacity factor activity was demonstrable in the extract

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Lack of Relation between the Receptors for IgA and IgG and Haptoglobin-Binding Capacity

Nine different sera belonging to haptoglobin types 1-1, 2-1 or 2-2 were tested by diffusion in gel against the electrophoretically separated alk ex T4 There was no relation between precipitates 1_{T4} 2_{T4} 3_{T4} and 4_{T4} and the haptoglobin type

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KINETICS OF LOCOMOTION OF HUMAN GRANULOCYTES IN A DOUBLE-FILTER SYSTEM

Effect of Activating Plasma with Zymosan and Casein

AKSEL SCHREINER and DAG VAULA

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Schreiner A & Vaula D Kinetics of locomotion of human granulocytes in a double filter system Effect of activating plasma with zymosan and casein Acta path microbiol scand Sect. C *88* 83-88 1980

A new modification of Boyden s millipore filter technique is described in which leukocytes are sandwiched between two filters and allowed to migrate in two directions This technique offers a better basis for comparison with mathematical models than does single filter methods Using this technique casein in low concentrations was leukokinetic in the absence of plasma but did not show leukotactic activity unless plasma was present Zymosan activated plasma was leukokinetic and strongly leukotactic

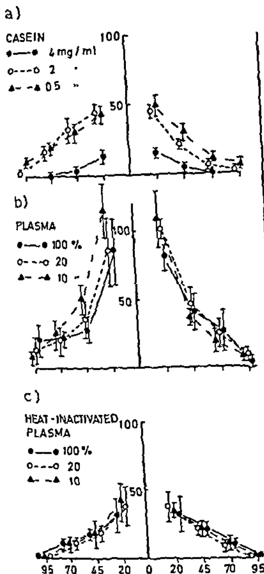
Key words *In vitro* chemotaxis leukokinesis leukotaxis zymosan casein

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Leukotaxins exert a chemotactic effect directly upon leukocytes whereas leukotaxigens need the presence of plasma serum or biological fluids or products [illegible] agent Demonstration of true chemotaxis can be done by choosing a method which allows the study of the behaviour of individual cells or comparison of the cell migration kinetics with a mathematical model designed to describe a directed locomotion (*Zigmond & Hirsch* 1973 *Rosen* 1976 *Zigmond* 1978) These conditions are not obtained with most variants of the Boyden millipore filter technique (*Boyden* 1962) widely used in the study of phagocyte migration The accumulation of cells in response to a gradient in most of these systems can be due to biased random migration or to chemotaxis (*Keller et al* 1977) Consequently information on agents alleged to be chemotactic to phagocytes on the basis of *Boyden* experiments is often uncertain (*Wilkinson* 1974) To improve the possibility of demonstrating directed migration of leukocytes in millipore filters and to be more able to distinguish between random and directed migration we developed another modification of the Boyden method in which leukocytes are sandwiched between two filters and thus allowed to move in two main directions The mathematical model used in a previous study (*Schreiner & Vaula* 1978) was modified to describe this new situation In the present study this method was used in experiments designed to test our previous finding that casein in the absence of plasma is not chemotactic but does increase random motility (*Schreiner & Vaula* 1978) Another pre[illegible] *Schreiner & Hopen* 1979) led us to examine also the influence of fresh heat inactivated and zymosan activated plasma on the migration of leukocyte populations in the double filter system

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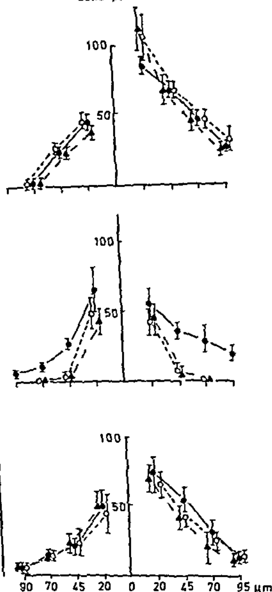


Fig 3 Distribution in double millipore filters after 60 min incubation of leukocytes exposed to a) casein b) fresh plasma and c) heat inactivated plasma under non gradient and gradient conditions

In contrast to our previous models (*Schreiner & Vaula* 1978) these models describe the distribution in infinite media corresponding to the experimental conditions used

Provided our simple model is adequate, the observed cell distribution under nongradient (»random mobility«) conditions should be comparable to equation (1) and the distribution under gradient (»chemotaxis«) conditions should be comparable to equation (2)

Experimental results All experimental results are shown in Fig 3, 4 and 5 The leukocytes were sandwiched between 2 millipore filters of 3 µm pore size, the filters with cells mounted in blind well

MATERIALS AND METHODS

Modified Boyden chambers were purchased from Neuroprobe, Bethesda, Md, USA, millipore filters with a pore size of 3 µm from Millipore Filter Corp, Bedford, Mass, USA, casein Hammarsten from Merck, Darmstadt, West Germany, and zymosan from Sigma, St Louis, Mo, USA Hanks' balanced salt solution with the addition of 2% human serum albumin (Hanks'/albumin) was used a main cell suspending medium

Blood Blood was donated by healthy *members of the* hospital staff, and by healthy medical students The blood was heparinised to contain 10 i u /ml The plasma used was always autologous

Separation of leukocytes Leukocytes were separated by dextran sedimentation and the contaminating erythrocytes lysed with ammonium chloride, as described previously (*Schreiner* 1978)

Activation of plasma with zymosan 100 mg washed zymosan in 10 ml plasma was incubated for 1 h at 37 °C The plasma was centrifuged 10 min at 1800 × *g*, and the supernatant used

Activation of plasma with casein Casein, 2 mg/ml or 0 5 mg/ml in fresh, autologous plasma was incubated for 1 h at 37 °C, and the mixture used

Inactivation of plasma Plasma was heated for 30 min at 56 °C

Modification of the Boyden technique The lower compartment of several chemotaxis chambers was filled with Hanks'/albumin One millipore filter was positioned in each chamber and 1 2 × 10⁶ leukocytes (0 6 ml of a cell suspension containing 2 0 × 10⁹/l leukocytes) were allowed to settle on the *filters by gravity* After 20 min the supernatant was pipetted off, the filters removed and pairs of filters carefully transferred to new *chambers* where they were assembled, the cell layers facing each other For gradient experiments the lower compartment of these chambers had already been filled with the appropriate attractant For non gradient experiments, the stimulant at a chosen concentration was present on both sides of the filters The chambers were incubated for the appropriate period at 37 °C in 100% relative humidity, thereafter the filters were removed, fixed in methanol and ethanol stained with hematoxylin dehydrated in ethanol, cleared with xylene and mounted on glass slides

Experimental design Gradient and non gradient experiments were made with casein in concentrations of *2 1 and 0 25 mg/ml dissolved in* Hanks'/albumin with fresh plasma, zymosan activated plasma and heat-inactivated plasma each in concentrations of 10 20 and 100% in Hanks'/albumin and with casein-activated plasma in concentrations of 1 and 0 25 mg/ml in Hanks'/albumin For each variable, 4 chambers were run in parallel The chambers were generally incubated for 60 min, and the filters immediately fixed The infilter distribution in each filter was determined by photographing 2 fields on each of 4 levels at 20 45, 70 and 95 micrometers respectively, from the cell starting side This procedure is described in detail elsewhere (*Schreiner* 1978)

RESULTS

Theoretical approach When particles move at *random in a homogeneous substrate* from an initial distribution represented by a thin layer in position x = 0 at time t = 0, the distribution at a later time will be Gaussian, as indicated previously (*Jacobs* 1967, *Rosen* 1976, *Schreiner & Vaula* 1978)

$$n(x,t) = (4\pi\mu t)^{-1/2} N \exp(-x^2/4\mu t) \qquad (1)$$

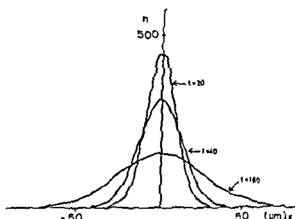


Fig 1 Theoretical distribution according to equation (1) for 3 values of t (min) of particles starting in position 0 and moving at random in an infinite medium Total number of particles per field (cross-sectional area) = 10^4, diffusion constant (µ) = 2 µm²min⁻¹

where µ = constant mobility for all particles at all values of x and t, N = constant number of particles per cross-sectional area

When a directional force is added to this simple model by assuming a constant velocity v, the solution to the transport equation is (*Jacobs* 1967, *Rosen* 1976, *Schreiner & Vaula* 1978)

$$n(x,t) = (4\pi\mu t)^{-1/2} N \exp(-(x-vt)^2/4\mu t) \qquad (2)$$

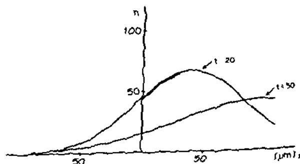
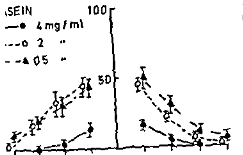
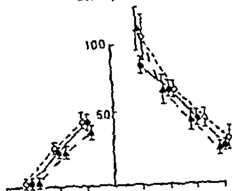
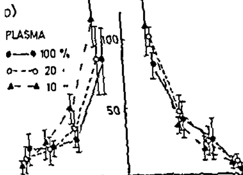
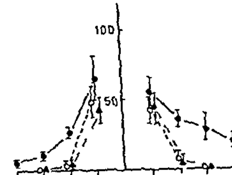


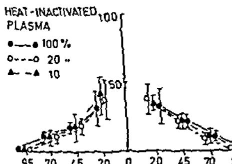
Fig 2 Theoretical distribution according to equation (2) for 2 values of t (min) of particles starting in position 0 and moving in an infinite medium when the mobility results from random *diffusion* and an additional, directed force Total number of particles per field (cross-sectional area) = 7520, diffusion constant (µ) = 50 µm²min⁻¹, velocity resulting from directed force (v) = 2µm min⁻¹











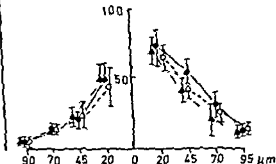


Fig 3 Distribution in double millipore filters after 60 min incubation of leukocytes exposed to a) casein, b) fresh plasma and c) heat inactivated plasma under non gradient and gradient conditions

In contrast to our previous models (*Schreiner & Vaula* 1978) these models describe the distribution in infinite media corresponding to the experimental conditions used

Provided our simple model is adequate the observed cell distribution under nongradient (random ...) conditions should be comparable to equation (1), and the distribution under gradient (chemotaxis) conditions should be comparable to equation (2)

Experimental results All experimental results are shown in *Fig 3, 4 and 5* The leukocytes were sandwiched between 2 millipore filters of 3 μm pore size, the filters with cells mounted in blind well

MATERIALS AND METHODS

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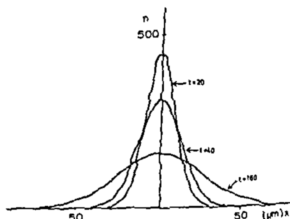


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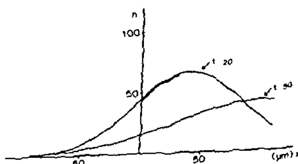


Fig 2 Theoretical distribution according *to equation (2)* for 2 values of t (min) of particles starting in position 0 and moving in an *infinite medium when the mobility* results from random *diffusion and an additional* directed force Total number of particles per field (cross sectional area) = 7520 diffusion constant (µ) = 50 µm²min⁻¹ velocity resulting from directed force (v) = 2µm min⁻¹

Boyden type chemotaxis chambers and incubated for 60 min In non gradient experiments the stimulant was present at equal concentration in both compartments In gradient experiments the stimulant was always present in the lower compartment and Hanks /albumin in the upper compartment The results from the upper filter are shown in a reversed manner to the left in each coordinate system the results from the lower filter to the right Each point on the curves represents the mean count of duplicate fields on each filter from 4 parallel experiments in each of 4 individuals (= 32 observations) ± SEM

DISCUSSION

The method used in the present study on leukocyte migration offers clear advantages By allowing cells to migrate in two main directions it yields a display of the total cell kinetics limited only by the thickness of the filter Under non-gradient conditions i e when the leukocytes are moving at random these kinetics are expected to be described by a normal distribution which is symmetrical with respect to the plane in which the leukocytes are layered In experiments with a gradient of a chemotaxin a normal distribution of cells is expected which moves towards the source of the gradient, a phenomenon familiar to all physicians from the pattern of protein electrophoresis (*Rosen* 1976 *Zigmond & Hirsch* 1978 *Schreiner & Vaula* 1978) This movement will result in a reduction of cell counts in the filter distant from the source of the gradient (hereafter called »the left filter«) This additional information is valuable since demonstration of the most discriminating part of the distribution in single filters which is close to the deposited cell layer (Fig 2 bold face line) is hampered by optical disturbances from this layer (*Schreiner* 1978) In experiments using an agent which is purely leukokinetic stimulation of random motility or increasing the incubation time – will result in a broadening and flattening of the distribution curve which however is still symmetrical In gradient experiments with a leukokinesin the leukocytes will accelerate as they move at random up the gradient and encounter increasing concentrations of the leukokinesin (*Schreiner* 1977) Consequently the cell distribution in filters from such experiments will be skewed some cells in the filter facing the source of the gradient (the right filter) having moved further However since previous results indicate that the number of cells responding to a locomotor stimulant may depend on the stimulant concentration (*Schreiner & Vaula* 1978) and the presented models imply that all particles start to locomote simultaneously comparison with the models requires great care In the casein experiments with a uniform casein concentration it is most probable that the highest casein concentration (Fig 3a) reduced the number of responding cells since the in filter cell count reached zero already at 70 µm For the same reason the quantitative cellresponse using heat inactivated plasma also appeared to be reduced (Fig 3c) However in contrast to the effect in capillary tubes heat inactivation of plasma did by no means abolish the random migration in millipore filters In non gradient experiments with casein concentrations lower than 2 mg/ml (Fig 3a) with zymosan activated (Fig 4a) and with casein activated plasma (Fig 4b) the random migration can carefully be considered as increased as compared to fresh plasma (Fig 3b) Generally modulation of migration under non gradient conditions by the factors tested was not very impressive Except for casein variation in concentration of the factors tested had little influence on the cell distribution Under gradient conditions modulation of the response varied from no influence to dramatic change With casein gradients the distribution was skewed in the direction of the right filter (Fig 3a) but did not compare with the model for directed migration The skewness may be explained by a larger number of cells responding in the filter facing the casein gradient and by increasing stimulation of random migration during passage of cells through the right filter The cell distribution of cells exposed to a gradient of fresh plasma remained symmetric indicating that the plasma was not chemotactic (Fig 3b) With heat inactivated plasma a small asymmetry occurred for which there is no ready at hand explanation (Fig 3c) A gradient of diluted zymosan activated plasma had a dramatic effect on the in filter cell distribution making it compare well with the model for directed migration (Fig 4a) This effect was approximately equal for 10 and 20% zymosan-activated plasma but far less pronounced when the activated plasma was used undiluted The shape of the distribution also compared favourably with the model for directed migration when different incubation times were used although the locomotor process seemed to be retarded with increasing time (Fig 5) The retardation can be explained either by the cells not starting simultaneously or by inhibition of motility as cells move up the gradient and encounter increasing concentrations of [illegible]

[illegible] the model for directional migration (Fig 4b) However the

NO GRADIENT

Cells per field

GRADIENT

Cells per field

a)

ZYMOSAN-ACTIVATED PLASMA

●——● 100 %

○----○ 20 "

▲— —▲ 10

b)

CASEIN-ACTIVATED PLASMA

●——● 2 mg/ml

▲— —▲ 0.5

95 70 45 20 0 20 45 70 95 90 70 45 20 0 20 45 70 95 μm

Fig 4 Distribution in double millipore filters after 60 min incubation of leukocytes exposed to a) zymosan activated plasma and b) casein activated plasma under non gradient and gradient conditions

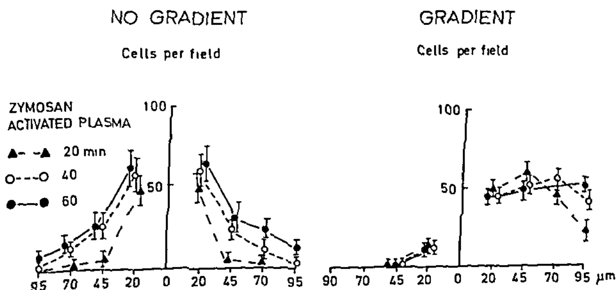


Fig 5 The distribution in double millipore filters of leukocytes exposed to zymosan activated plasma at 3 different incubation times under non gradient and gradient conditions

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LEUKOCYTE MIGRATION IN DIFFERENT SYSTEMS

Effect of Colchicin Kinetics of Migration under Agarose

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Schreiner A Kalager T & Vaula D Leukocyte migration in different systems Effect of colchicin Kinetics of migration under agarose Acta path microbiol scand Sect. C 88 89-96 1980

In a double filter modification of Boydens method formylmethionylleucylphenylalanine (FMLP) needed the presence of fresh plasma to induce leukocyte locomotor kinetics which compared with a model for directional migration FMLP or zymosan activated plasma did not stimulate migration in capillary tubes The kinetics of leukocyte migration under agarose towards zymosan activated plasma were more compatible to a model for increased random motility than for directional migration Treatment of leukocytes with colchicin reduced their migration under gradient and non gradient conditions in millipore filters capillary tubes and in under agarose experiments

Key words Leukocyte migration capillary tubes millipore filters under agarose

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The knowledge of what is actually being measured with available *in vitro* methods for measuring chemotaxis is insufficient In a previous study we demonstrated that the influence of glass adhesion inhibits leukocyte migration in capillary tubes (*Schreiner & Hopen* 1979) but also other physical and chemical influences may render results obtained with different methods incompatible Also the differentiation between random and directed migration in these test systems is difficult

In Boyden filter experiments (*Boyden* 1962) using gradients of casein we were unable to demonstrate leukocyte locomotion kinetics which compared to a mathematical model for directed migration (*Schreiner & Vaula* 1978) However in our double filter modification of the Boyden system plasma activated with casein or zymosan induced kinetics which were comparable to the model Other chemotactic factors have not been tested with this method

Differentiation between directed and random migration is routinely carried out when the under agarose technique (*Nelson et al* 1975) is used The non-symmetric pattern of leukocyte migration in this assay is despite the lack of evidence seen as an expression of directed migration

Antitubulins especially colchicin have been of importance for the concept of random and directed migration since the report by *Gallin et al* (1978) that directed migration was inhibited by this agent whereas random migration was not influenced

To study the influence of some chemotactic factors on leukocyte migration in different test systems we compared the results with formylmethionylleucylphenylalanine (FMLP) in Boyden filter experiments and capillary tube experiments The effect of zymosan activated plasma was examined in capillary tubes and in under agarose experiments Furthermore we examined the influence of FMLP on the leukocyte migration kinetics in millipore filters in the presence and absence of fresh or heat inactivated plasma In under agarose experiments we compared the leukocyte locomotion kinetics with computer-simulated mathematical models for random and directed migration Finally, we investigated the influence of colchicin on the leukocyte migration in Boyden filter experiments capillary tubes and under-agarose experiments

relatively high cell counts in the left filter indicated a considerable contribution of random migration to the total distribution

The following conclusions can be drawn from our study When the leukocyte distribution in millipore filters in comparison with mathematical models is used in the study of leukocyte locomotion the presented double filter method gives more reliable results than single filter methods The advantages include the display of a greater part of the distribution and a better distinction between random and directional migration The results support previous findings that casein is not – or only weakly – chemotactic However casein is capable of inducing chemotactic activity in plasma

In contrast to the findings with capillary tubes random migration in millipore filters is not dependent on a heat labile plasma factor

Activation of complement with zymosan renders plasma highly chemotactic These findings support the assumption that activated complement is the major chemotactic principle in plasma i e under physiological conditions (*Snyderman et al* 1971, *Wilkinson et al* 1973) Finally our results emphasize the obligation to employ a method which unequivocally demonstrates directed migration before proclaiming chemotactic factors

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This study was supported by grants from the *Norwegian Research Council for Science and the Humanities* from *L Meltzers Høyskolefond* and from the *Norwegian Cancer Society*

The skilled technical assistance by *Britt Edvardsen* and *Ole Ingar Paulsen* is thankfully acknowledged

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RESULTS

ion gradient Boyden millipore experiments with 10^{-6}M FMLP combined with 20% fresh plasma in h compartments migration was slightly increa as when compared to experiments with FMLP the absence of plasma In gradient experiments h FMLP migration was markedly increased en plasma was present (Fig 1a) When the same es of experiments were performed with FMLP in presence and absence of heat inactivated plasma noticeable difference in migration was observed g 1b)

In capillary tube experiments the presence in the l suspending plasma of 10^{-8} and 10^{-7}M FMLP l not influence leukocyte migration as compared controls ($p > 0\ 1$) whereas 10^{-6}M FMLP luced migration significantly ($p < 0\ 05$) Zymo-n-activated plasma diluted in fresh plasma reduced tube migration of leukocytes ($p < 0\ 05$) when the concentration reached 10%, but not ($p > 0\ 1$) at 1% or lower (Fig 2)

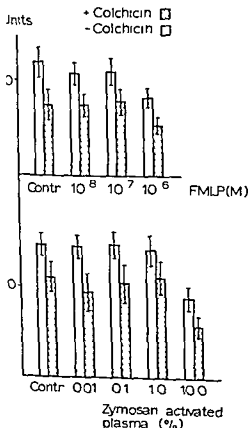


Fig 2 Migration in capillary tubes of colchicin treated (n 7) and untreated (n 6) leukocytes Leukocytes suspended in fresh plasma with the addition of FMLP upper panel) Leukocytes suspended in zymosan activated plasma (lower panel) Leukocytes suspended in fresh undiluted plasma (contr)



Fig 3 Migration after 180 min incubation of colchicin treated and untreated leukocytes towards (right panel) and away from (left panel) undiluted zymosan-activated plasma n = 9 Mean migrated distance ± SEM

Migration of colchicin treated and untreated leukocytes was tested in all three systems and compared to migration of cells that had been left untreated In millipore filter experiments treatment with colchicin reduced leukocyte migration in both gradient and non gradient experiments The in filter counts were lower for colchicin treated cells in each field on each level in all filters from three experiments (Fig 1c) In capillary tubes (Fig 2) colchicin treatment reduced leukocyte migration significantly ($p < 0\ 01$) A reduction in migration of colchicin treated cells in capillary tubes was also observed when FMLP or zymosan activated plasma was present In under agarose experiments colchicin treatment reduced leukocyte migration towards ($p < 0\ 05$) as well as away from ($p < 0\ 05$) zymosan activated plasma (Fig 3)

In some under agarose experiments using zymosan activated plasma as stimulant and 5 periods of incubation (40 90 160 250 and 360 min) the kinetics of leukocyte migration was determined A counting grid with boxes each comprising a true area of 200 × 100 μm (1 unit area) was superimposed on photographs (magnification 500) of cells that had migrated (Fig 4) All images of cells visible within each box were counted in the direction towards the stimulant, as well as away from it. The mean counts in corresponding boxes from 3 experiments were calculated and plotted in a coordinate system against the distance from the well to the lateral demarcation of the appropriate box (1 unit = 100 μm) (Fig 5)

To be able to compare these findings with mathematical models we developed a theoretical approach

Theoretical approach to leukocyte migration under agarose Rosen (1976) suggested a transport theory for neutrophils during chemotaxis in millipore filters of the following form

$$n(x\ t) = (4\pi\mu t)^{-1/2} N (\exp -(x - vt)^2/4\mu t) \quad (1)$$

MATERIALS AND METHODS

Blood was drawn from members of the hospital staff and medical students and heparinised to contain 10 i u per ml N-formylmethionylleucylphenylalanine (FMLP) was purchased from Sigma, St Louis, Mo, USA, colchicin from Merck, Darmstadt, West Germany, tissue medium 199 and HEPES buffer from Flow laboratories, bovine serum albumin from Armour Pharmaceutical Co, Ltd, Eastbourne, UK, agarose from Litex, Glostrup, Denmark and tissue culture dishes (No 3002, 60 × 15 mm) *from Falcon Plastics, Oxnard, Calif, USA* All other materials were obtained as stated previously (*Schreiner & Hopen* 1979) Hanks' balanced salt solution (HBSS) was used as the main cell suspending medium

Separation of leukocytes Leukocytes were isolated by dextran sedimentation and the contaminating erythrocytes lysed with ammonium chloride as described previously (*Schreiner 1978*)

Activation of plasma Plasma was activated with zymosan as described previously (*Schreiner & Vaula 1980*)

Colchicin treatment Isolated leukocytes suspended in a solution of 10^{-6}M colchicin in HBSS were incubated for 30 min at 37 °C immediately prior to each experiment After incubation the leukocytes were washed once in heparinised (2 5 i u per ml) physiological saline and resuspended in HBSS supplemented with human or bovine serum albumin (HBSS/albumin)

Millipore filter migration A modification of Boyden's method (*Schreiner & Vaula* 1980) was used Briefly leukocytes were sandwiched between two millipore filters (pore size 3 μm) and each pair of filters mounted in blind-well, Boyden type chemotaxis chambers For gradient experiments, the test substance was present in the lower compartment and HBSS supplemented with 2% (v/v) human serum albumin in the upper compartment For non-gradient experiments the test substance in *HBSS/albumin was present at equal concentrations in* both compartments The chambers were incubated in 100% humidity at 37 °C for 60 min The kinetics of leukocyte migration in these experiments were determined by photographic in-filter counting at different distances from the cell-starting side of the filter (*Schreiner* 1978)

Tube migration Migration of leukocytes *in capillary* tubes was studied using the method of *Ketchel & Favour* (1955) in our modification (*Schreiner & Hopen* 1979) Briefly a suspension of 7 × 10^9 leukocytes per l in plasma or in plasma containing a test substance was aspirated half way into a hematocrit tube, the tube sealed at the opposite end and centrifuged at 2500 × *g* for 3 min The tubes were incubated at *37 °C for 3 h* and the distance migrated by the leading front of leukocytes from the demarcation of the cell button up the inside wall of the tube was measured in units (72 units = 1 mm) with an ocular micrometer

Under-agarose migration For leukocyte migration [illegible] dishes were pre-coated with gelatin by wetting [illegible] with an 0 5% (v/v) aqueous solution of gelatin and allowed to dry Furthermore, the cell suspending medium (HBSS) was supplemented with 2% (w/v) bovine serum albumin Briefly, in each hardened agarose plate, 18 wells with a diameter of 2 5 mm were punched out The wells were arranged with three in each of six radiuses, the center-to-center distance between the wells in each radius being *5 mm The outer well in each radius* received the attractant (undiluted zymosan activated plasma), the middle well received a suspension of mixed leukocytes (adjusted to 1 5 × 10^{10} granulocytes per l), and the inner well received a control medium (HBSS with 2% bovine albumin) After incubation in 100% humidity at 37 °C, the agarose and cells were fixed with methanol and formaldehyd, the agarose layer carefully removed, the cells stained with hematoxylin and pictures of the cells projected on a screen where the maximal distance of migration towards, as well as away from the stimulant could be measured according to *Nelson et al* (1975)

Statistical method The Wilcoxon rank sign test for paired samples was used

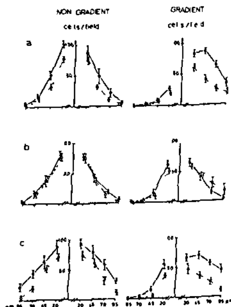


Fig 1 In filter distribution of leukocytes in Boyden double filter experiments made under *non gradient* and gradient conditions

a) Cells suspended in HBSS/albumin --------
fresh plasma ——

b) Cells suspended in HBSS/albumin -- -----
heat inactivated plasma ——

c) Cells treated with colchicin --------
not treated ——

In the non gradient experiments 10^{-6} M FMLP was present in both compartments In the gradient experiments, 10^{-6} FMLP was present *only in the lower* compartment

RESULTS

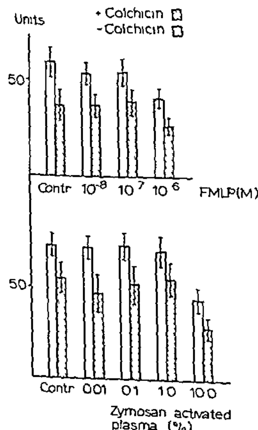
In non gradient Boyden millipore experiments with 10^{-6}M FMLP combined with 20% fresh plasma in both compartments migration was slightly increased as when compared to experiments with FMLP in the absence of plasma In gradient experiments with FMLP migration was markedly increased when plasma was present (Fig 1a) When the same types of experiments were performed with FMLP in the presence and absence of heat inactivated plasma no noticeable difference in migration was observed (Fig 1b)

In capillary tube experiments the presence in the cell suspending plasma of 10^{-8} and 10^{-7}M FMLP did not influence leukocyte migration as compared to controls ($p > 0.1$) whereas 10^{-6}M FMLP reduced migration significantly ($p < 0.05$) Zymosan-activated plasma diluted in fresh plasma reduced tube migration of leukocytes ($p < 0.05$) when the concentration reached 10%, but not ($p > 0.1$) at 1% or lower (Fig 2)



Fig 3 Migration after 180 min incubation of colchicin treated and untreated leukocytes towards (right panel) and away from (left panel) undiluted zymosan activated plasma n = 9 Mean migrated distance ± SEM

Migration of colchicin treated and untreated leukocytes was tested in all three systems and compared to migration of cells that had been left untreated In millipore fi



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field on each level in all filters from three experiments (Fig 1c) In capillary tubes (Fig 2) colchicin treatment reduced leukocyte migration significantly ($p < 0.01$) A reduction in migration of colchicin treated cells in capillary tubes was also observed when FMLP or zymosan activated plasma was present In under agarose experiments colchicin treatment reduced leukocyte migration towards ($p < 0.05$) as well as away from ($p < 0.05$) zymosan-activated plasma (Fig 3)

In some under agarose experiments using zymosan activated plasma as stimulant and 5 periods of incubation (40 90 160 250 and 360 min) the kinetics of leukocyte migration was determined A counting grid with boxes each comprising a true area of 200 × 100 µm (1 unit area) was superimposed on photographs (magnification 500) of cells that had migrated (Fig 4) All images of cells visible within each box were counted in the direction towards the stimulant as well as away from it The mean counts in corresponding boxes from 3 experiments were calculated and plotted in a coordinate system against the distance from the well to the lateral demarcation of the appropriate box (1 unit = 100 µm) (Fig 5)

To be able to compare these findings with mathematical models we developed a theoretical approach

Theoretical approach to leukocyte migration under agarose Rosen (1976) suggested a transport theory for neutrophils during chemotaxis in millipore filters of the following form

$$n(x\ t) = (4\pi\mu t)^{-½} N(\exp -(x\ vt)^2/4\mu t) \quad (1)$$

MATERIALS AND METHODS

Blood was drawn from members of the hospital staff and medical students and heparinised to contain 10 i u per ml N formylmethionylleucylphenylalanine (FMLP) was purchased from Sigma St Louis Mo USA colchicin from Merck Darmstadt West Germany tissue medium 199 and HEPES buffer from Flow laboratories bovine serum albumin from Armour Pharmaceutical Co , Ltd Eastbourne UK agarose from Litex Glostrup Denmark and tissue culture dishes (No 3002 60 × 15 mm) from Falcon Plastics Oxnard Calif USA All other materials were obtained as stated previously (*Schreiner & Hopen* 1979) Hanks balanced salt solution (HBSS) was used as the main cell suspending medium

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Activation of plasma Plasma was activated with zymosan as described previously (*Schreiner & Vaula* 1980)

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Tube migration Migration of leukocytes in capillary tubes was studied using the method of *Ketchel & Favour* (1955) in our modification (*Schreiner & Hopen* 1979) Briefly a suspension of 7 × 10^{9} leukocytes per l in plasma or in plasma containing a test substance was aspirated half way into a hematocrit tube the tube sealed at the opposite end and centrifuged at 2500 × g for 3 min The tubes were incubated at 37 °C for 3 h and the distance migrated by the leading front of leukocytes from the demarcation of the cell button up the inside wall of the tube was measured in units (72 units = 1 mm) with an ocular micrometer

Under agarose migration For leukocyte migration under agarose we used the method presented by *Nelson et al* (1975) as modified by *Forsgren & Schmeling* (1977) with the additional modification that the tissue culture dishes were pre coated with gelatin by wetting the dishes with an 0 5% (v/v) aqueous solution of gelatin allowed to dry Furthermore the cell suspend medium (HBSS) was supplemented with 2% (w bovine serum albumin Briefly in each hardened agar plate 18 wells with a diameter of 2 5 mm were punc out The wells were arranged with three in each of radiuses the center to-center distance between the w in each radius being 5 mm The outer well in each rac received the attractant (undiluted zymosan activa plasma) the middle well received a suspension of mi leukocytes (adjusted to 1 5 × 10^{10} granulocytes per and the inner well received a control medium (HE with 2% bovine albumin) After incubation in 10(humidity at 37 °C the agarose and cells were fixed w methanol and formaldehyd the agarose layer carefu removed the cells stained with hematoxylin and pictu of the cells projected on a screen where the maxir distance of migration towards as well as away from stimulant could be measured according to *Nelson et* (1975)

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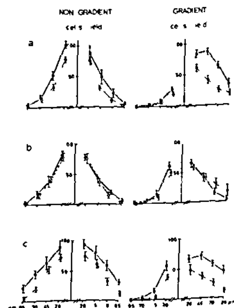


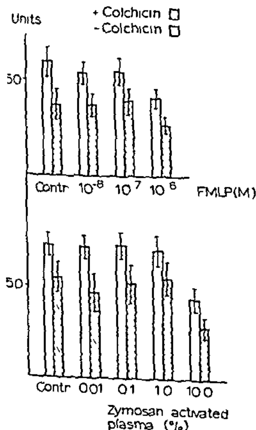
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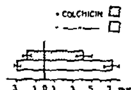


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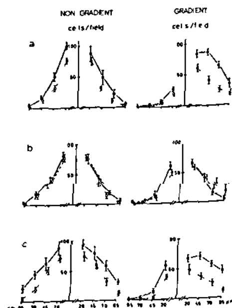


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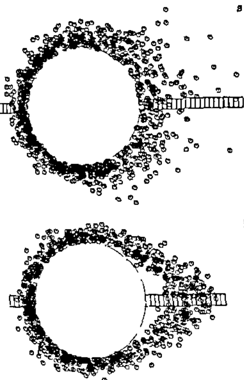


Fig 6 Computer simulated distribution of particles according to the equations (2) (3) (4) and (5) after one selected period of time (160 min) Consecutive areas of 100 × 200 µm are indicated
a) Distribution results from stimulated random mobility alone *i e* $\chi = 0$
b) Distribution results from constant random mobility plus a directional flux *i e* $\beta = 0$
To avoid overloading of the illustration the initial number N of particles is reduced to 1000 For technical reasons each particle is drawn with a considerable size

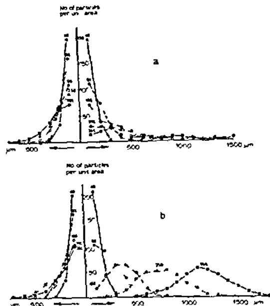


Fig 7 Computer-simulated particle concentration in consecutive areas (100 × 200 µm) based on equations (2) (3) (4) and (5) Each point on the curves represents the particle density in one area plotted against the shortest distance from the starting configuration of the particles to the lateral demarcation of the appropriate area Each curve represents the distribution of densities from all areas after one of 5 periods of time (min) The arrows indicate movement away from (←) and towards (→) the source of the locomotor stimulant
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To imitate the under agarose experiments the particle density in areas of 100 × 200 µm each corresponding to one box of the counting grid used in the experiments (Fig 4) was counted by the computer The simulated particle distribution after 160 ... [illegible] ... number of particles ... [illegible] ... as shown in Fig 7a and b

Comparison of the curves resulting from the experimental results (Fig 5) with the curves based on the models (Fig 7a and b) shows that the experimental results are better comparable to the model in which the particle distribution is a consequence of increased random motility alone (Fig 7a) This indicates that the migration of leukocytes under agarose towards a »chemotaxin« may be the result of stimulated random motility rather than of directed migration

DISCUSSION

Insufficient knowledge of the mechanism of leukocyte locomotion hampers the interpretation of results obtained with *in vitro* methods for measuring such locomotion The different conditions given to the leukocytes in various *in vitro* assays make it probable that different functions are being

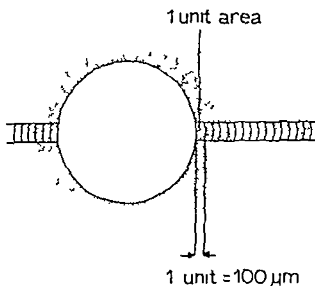


Fig 4 Sketch of leukocytes migrating under agarose and superimposed counting grid

where μ is the random diffusion coefficient of particles (leukocytes) N the initial number of particles and v the velocity induced upon the particles by the chemotactic force To be able to computer simulate the kinetics of leukocytes migrating under agarose we performed a stochastic reformulation of this model In our version the particles possess equal time independent motility

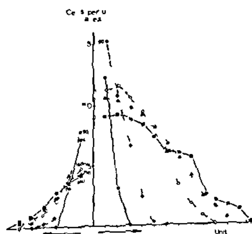


Fig 5 Distribution in selected areas (boxes of counting grid) of leukocytes having migrated for different periods of time under agarose Each point on the curves represents the count from one box (mean of 3 experiments) plotted against the shortest distance from the periphery of the cell containing well to the lateral demarcation of that box Each curve represents the mean distribution of counts from all boxes after one of 5 ... (m n) The arrows indicate ...) *zymosan* ... he counts in ... % of the mean ranged from 5 to 105 ... averaged 67

that is their locomotive capacity remains unchanged The displacement of each particle Δr in a sufficiently limited time interval Δt has two components the resultant of which being the vector

$$\Delta r = \Delta r_r + \Delta r_d \quad ($$

Δr_r is a random (Brownian) component with two-dimensional Gaussian distribution

$$f(\Delta r_r) = (4 \pi \mu t)^{-1} \exp(-(\Delta r_r)^2/4 \mu \Delta t) \quad ($$

Δr_d is a chemotactic vector determined by the concentration c of the locomotor stimulant

$$\Delta r_d = \chi \nabla c \Delta t \quad ($$

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$$\mu = \mu_0 + \beta c \quad ($$

where β is the influence of the locomotor stimulant on the random mobility of each particle (leukokinetic coefficient)

We also assumed that the initial concentration the locomotor stimulant c_o the absolute value which is set to 1 is the same in all experiments and that this concentration decays according to a two dimensional Gaussian distribution Furthermore we assumed that the locomotor stimulant has constant diffusion coefficient D in all locations particle movement

The computer simulation of this model is quite simple using a high capacity computer and subroutine which generates pseudorandom numbers After having chosen realistic empirical obtained values for μ_o β and D a start-configuration is generated according to the assumed distribution of N particles at t = 0 Subsequently each particle is given a small displacement Δr corresponding to a time step Δt and to the equations (and (3) A very small time step Δt is chosen so that the change in particle distribution and the change the concentration profile of the locomotor stimulant are insignificant as compared to the total change during an under agarose experiment This procedure is repeated M times making the total time t = MΔt correspond to one incubation period in the under agarose experiments

In our study simulations were performed for periods of time corresponding to the incubation times used in the under agarose experiments (40 9

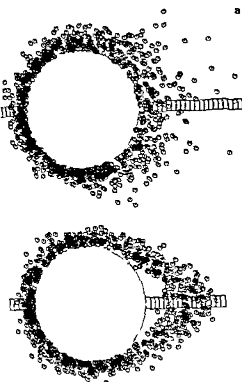


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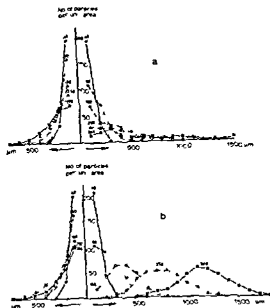


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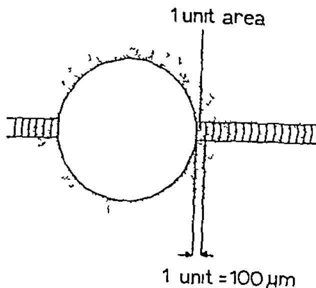
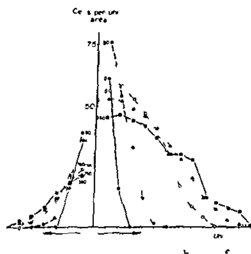


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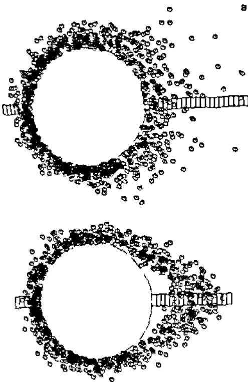


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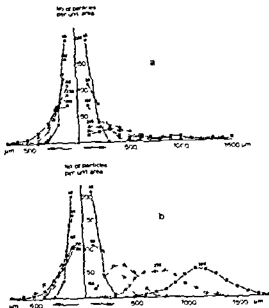


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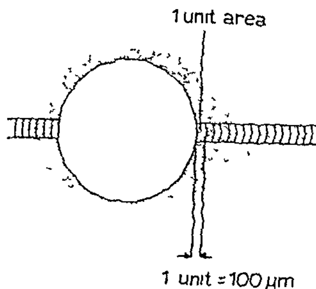


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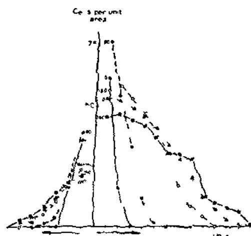


Fig 5 Distribution in selected areas (boxes of counting grid) of leukocytes having migrated for different periods of time under agarose Each point on the curves represents the count from one box (mean of 3 experiments) plotted against the shortest distance from the periphery of the cell-containing well to the lateral demarcation of that box Each curve represents the mean distribution of counts from all boxes after one of 5 different incubation periods (min) The arrows indicate movement away from (←) and towards (→) zymosan activated plasma The standard deviation of the counts in % of the mean ranged from 5 to 105 and averaged 67

that is their locomotive capacity remains changed The displacement of each particle Δr sufficiently limited time interval Δt has components, the resultant of which being the ve

$$\Delta r = \Delta r_r + \Delta r_d$$

Δr_r is a random (Brownian) component with two-dimensional Gaussian distribution

$$f(\Delta r_r) = (4\pi\mu t)^{-1}\exp(-(\Delta r_r)^2/4\mu\Delta t)$$

Δr_d is a chemotactic vector determined by concentration c of the locomotor stimulant

$$\Delta r_d = \chi\nabla c\Delta t$$

where χ is the chemotactic flux coefficient and ∇c the two-dimensional gradient of c. Both μ and may depend in a general way on c However we simplified the model by assuming that χ independent of c and that μ varies linearly with c

$$\mu = \mu_0 + \beta c \quad (3)$$

where β is the influence of the locomotor stimulant on the random mobility of each particle (leukokinetic coefficient«)

We also assumed that the initial concentration of the locomotor stimulant c_0, the absolute value of which is set to 1, is the same in all experiments and that this concentration decays according to a two-dimensional Gaussian distribution Furthermore, we assumed that the locomotor stimulant has a constant diffusion coefficient D in all locations of particle movement

The computer simulation of this model is quite simple using a high capacity computer and a subroutine which generates pseudorandom numbers After having chosen realistic empirically obtained values for μ_0, β and D a start-configuration is generated according to the assumed distribution of N particles at $t = 0$ Subsequently each particle is given a small displacement Δr corresponding to a time step Δt and to the equations (2) and (3) A very small time step Δt is chosen so that the change in particle distribution and the change in the concentration profile of the locomotor stimulant are insignificant as compared to the total changes during an under-agarose experiment This procedure is repeated M times making the total time $t_M = M\Delta t$ correspond to one incubation period in the under-agarose experiments

In our study simulations were performed for 5 periods of time corresponding to the incubation times used in the under-agarose experiment (40 90

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In capillary tube experiments the leukocytes move under non gradient conditions and their *mode of migration must therefore be considered* to be random In our tube experiments migration was not increased by FMLP in concentrations which are known to significantly stimulate leukocyte locomotion in glass slide experiments (*Zigmond* 1978) and in millipore filter experiments (*Schiffman et al* 1975) On the contrary in a higher concentration *e g* 10^{-6}M *FMLP reduced the distance of migration* significantly Also zymosan activated plasma reduced leukocyte migration in capillary tubes when the concentration was 10% whereas lower concentrations had no influence We have no explanation for the lack of stimulatory effect of these factors on leukocyte tube migration However the findings clearly show the non homogeneity *of results* obtained with different methods The inhibitory effect of *locomotion stimulants on* leukocyte tube migration may be explained by the leukocyte aggregating effect of high concentrations of these factors (*O Flaherty et al* 1975)

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Given that the locomotor reaction of leukocytes to a uniform concentration of a stimulant is qualitatively different from the locomotor reaction of leukocytes when exposed to a gradient of the same stimulant *in vitro* chemotaxis assays should be able to detect this difference Using casein as a stimulant of locomotion and comparison of the leukocyte migration kinetics with mathematical models we were able to demonstrate such difference, but unable to attribute the difference to a directional type of migration (*Schreiner & Vaula* 1978) However using a new double-filter variant of Boyden s method (*Boyden* 1962) with gradients of casein in the presence of plasma and with zymosan-activated plasma, leukocyte in-filter distributions were obtained which compared well to the model for directed migration (*Schreiner & Vaula* 1980) In the present study, we demonstrated that also FMLP, which is accepted as a strong chemotaxin (*Schiffman et al* 1975), needed the presence of plasma to induce a migrational pattern on the leukocytes which was comparable with the mathematical model This effect did not occur when FMLP was combined with heat inactivated plasma The moderate asymmetry of the in filter distribution in double filters obtained with gradients of FMLP in the absence of plasma may be explained by increase in leukocyte random motility as the leukocytes move towards the source of the gradient Our results with FMLP did not confirm that this agent is a strong locomotor stimulant in the absence of plasma The finding of an increased migration of leukocytes when FMLP is combined with fresh plasma, but not with heat inactivated plasma indicates that the increased migration may be due to activation of complement

The kinetics of leukocytes demonstrated in millipore filters reflect the importance of complement in stimulating leukocyte locomotion but cannot without reservation be accepted as evidence for directed migration In membrane filters such as the millipore type, the pores run fairly straight through the filter showing a tortuosity coefficient of only 1 2–1 4 (*Ren* 1976) Under such conditions it will depend on the mechanism by which leukocytes sense and react to locomotor stimulants whether they have the liberty of choosing to migrate towards the source of the gradient, or away from it If the leukocytes have no liberty to move away from the gradient source, a directional movement will be forced upon the cell population A possible interpretation of the different degrees of asymmetry in leukocyte distribution when different factors are tested, is that they may reflect only different degrees of random motility, the highest of which being induced by activated complement However since the random migration in non gradient experiments was not impressingly increased by any of the factors, the greater part of the directional component would have to be explained by influence of the filter structure

In capillary tube experiments, the leukocytes move under non-gradient conditions, and their mode of migration must therefore be considered to be random In our tube experiments migration was not increased by FMLP in concentrations which are known to significantly stimulate leukocyte locomotion in glass slide experiments (*Zigmond* 1978) and in millipore filter experiments (*Schiffman et al* 1975) On the contrary, in a higher concentration e g 10^{-6}M, FMLP reduced the distance of migration significantly Also zymosan activated plasma reduced leukocyte migration in capillary tubes when the concentration was 10%, whereas lower concentrations had no influence We have no explanation for the lack of stimulatory effect of these factors on leukocyte tube migration However, the findings clearly show the non-homogeneity of results obtained with different methods The inhibitory effect of locomotion stimulants on leukocyte tube migration may be explained by the leukocyte aggregating effect of high concentrations of these factors (*O Flaherty et al* 1975)

Microtubules have been thought to be of selective importance to directed migration of leukocytes since leukocytes treated with an antitubulin such as colchicin, have been reported to lose their ability to maintain directional movement, whereas they still moved at random (*Gallin et al* 1978 *Wilkinson & Allan* 1978) In the present study treatment of leukocytes with colchicin considerably reduced their migration in millipore filters under non gradient as well as under gradient conditions In capillary tubes in which the locomotor stimulant concentration is uniform, treatment with colchicin reduced leukocyte migration significantly The presence of chemo-

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No mathematical model designed to describe biological events can be accepted without reservation The particles in our computer simulated model move in steps rather than continously The parameters for diffusion and directed flux are chosen empirically Since in the experiments the leukocytes tended to congregate at the periphery of the cell containing well the particles in the model were started simultaneously at the periphery of the simulated well without being given the option to return to the well This last restriction may be an explanation for the difference between simulated particle concentration and experimental cell concentration near the starting point (well) The inherent uncertainties of a model such as the one presented makes it necessary to be careful in drawing conclusions However even the most critical interpretation indicates that the *a priori* acceptance of a non-symmetric cell distribution in under agarose experiments as an expression of directional leukocyte migration is not justified

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THE CELLULAR REACTION IN NORMAL AND TUMOUR-BEARING MICE FOLLOWING INTRAPERITONEAL GLUCAN INJECTION

R OLSTAD and R SELJELID

Inst tute of Medical Biology Department of Morphology University of Tromsø Tromsø Norway

Olstad R & Seljelid R The cellular reaction in normal and tumour bearing mice following intraperitoneal glucan injection Acta path microbiol scand Sect C *88* 97–101 1980

The effect of a single intraperitoneal injection of yeast glucan in C_3D_2 mice was studied The optimal dose for the induction of a cellular exudate was 0 4 mg/kg producing $17\ 6 \times 10^6$ cells on day 7 after injection The number of T-cells B-cells and macrophages in this exudate was analysed by the use of morphology immunofluorescence and the non specific esterase staining An increase in both T-cells B cells and macrophages was found after glucan injection There was an 8 fold increase in macrophages but the increase lasted only 14 days The increase in T-cells was smaller but lasted at least 35 days The effect of pretreatment with glucan on the development of an inoculated syngeneic methyl cholanthrene induced sarcoma was studied No effect on tumour take could be demonstrated but d fferences in T cell B-cell and macrophage content of the developing tumour were found A striking feature was a prolonged increase in tumour T-cell content

Key words Glucan macrophage mouse tumour

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Since *Riggi & DiLuzio* in 1961 (18) isolated the active stimulant of the reticuloendothelial system in zymosan – a high molecular weight β1–3 glucan commonly referred to as ›glucan‹ – much interest has been assigned to the biological properties and especially to the antitumour effect of this carbohy drate

The antitumour effect has been demonstrated in many experimental tumour systems in animals such as Shay myelogenic leukaemia in rats (5) ascitic sarcoma 180 (14) L 1210 leukaemia (3) and M 109 lung carcinoma in mice (20) Glucan also induced regression when injected into different

responsible for this effect is unknown

To elucidate this problem we have studied the cellular composition of exudate induced by intrape ritoneal injection of glucan We have also tested the effect against a syngeneic ascites tumour and have estimated the cellular composition in peritoneum at different stages after injection of ascitic tumour in mice pretreated with glucan

MATERIALS AND METHODS

Mice

Male $C_3D_2F_1$ hybrids ($C_3H/TifF$ ♀ × DBA/2 ♂) 8–10 weeks old (20–24g) at the start of the experiments were used for both tumour maintainance and for glucan experiments

Tumour

A syngeneic methylcholanthrene induced sarcoma MC1M (10 11 19) was used in all the experiments The

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AA subline grows in an ascites form when inoculated in doses higher than 10^5 viable tumour cells With lower inoculation doses some animals develop a solid tumour at the site of the inoculum probably because of leakage of tumour cells after inoculation The tumour is weakly immunogeneic judged by the fact that inoculation of as few as 5×10^2 viable cells give progressive solid tumour growth in approximately 80% of the animals The tumour was maintained in the ascites form by weekly transfer of 0 5–1 ml of ascites

Glucan

A β1–3 yeast cell wall glucan was the gift of Professor *Jan Raa* University of Tromsø Norway

The glucan was suspended in phosphate buffered saline (PBS) in a concentration of 1 mg/ml and sonicated with a MSE sonicator for 45–60 seconds at medium power The stock solution was sterilized by boiling and stored at 4 °C If stored for more than a week the suspension was resonicated before use Appropriate dilutions of the stock solution were made prior to injection so that the injected volume was constant

Chemicals

The medium used for cell washing was Eagles minimum essential medium supplemented with Hepes buffer (MEM Hepes) obtained from Gibco Grand Island New York USA Heparin was purchased from Nyegaard & Co Oslo Norway rabbit anti mouse brain antibody from Cedarlane Ltd Wellington Rd S London Canada rabbit immunoglobulin from Mann Research Laboratories Inc New York USA tetrame thyl rhodamine isothiocyanate (TRITC) from Baltimore Biological Lab Cockeyesville Maryland USA fluore scein isothiocyanate (FITC) from Koch Light Lab Colnbrook Buckinghamshire England human IgG from Kabi Stockholm Sweden sepharose from Phar macia Fine Chemicals Uppsala Sweden and staining solutions for May Grunwald Giemsa staining from Merck Darmstadt West Germany For nonspecific esterase staining basic fuchsin was obtained from Koch Light Lab α naphtyl acetate from Sigma Chemical Co St Louis Maryland USA and methyl green from Difco Lab Surrey England

Antibodies

The FITC F(ab) goat anti mouse Ig was produced purified and conjugated with fluorochromes as described by *Hannestad & Gaudernack* (9) Sheep anti rabbit Ig was produced by immunization of sheep with rabbit Ig precipitated with 50% saturated ammonium sulphate purified by binding to and elution from rabbit IgG linked to sepharose and conjugated with TRITC as described by *Bergqvist & Nilsson* (1) All antibodies were inactivated at 56 °C for 30 minutes and diluted in MEM containing 10 mg/ml human IgG aggregated at 63 °C for 10 minutes to block the Fc receptors of the cells The optimal dilutions of each antibody were titrated separa tely

Collection of Cells

Animals were killed by overdoses of ether and peritoneal exudate cells (PEC) or ascites tumour suspen sions were collected by aspiration following the injectio of 2 ml phosphate buffered saline with heparin (15 IU ml) Cells were counted in a Burker haemacytometer A cell suspensions were washed with MEM Hepes Viab lity of cells was tested by trypan blue exclusion Th viability of PEC was usually higher than 95% Th tumour cell viability decreased to about 85% within few hours of collection but then remained constan throughout the immunofluorescence procedure

Identification of Cells

Theta positive cells (T cells) and surface Ig positiv cells (B cells) were identified by immunofluorescence a follows Suspensions of PEC or ascites cells from tumour bearing mice were collected and washed twic with MEM hepes Tumour cell suspensions which wer visibly bloody were excluded from the experiments Cell were counted and 10^6 cells were incubated at 4 °C for 60 minutes with 50 µl rabbit anti mouse brain antibody They were then incubated with 50 µl FITC $F(ab')_2$ goat anti mouse Ig for 30 minutes at 4 °C Finally the cells were incubated with 50 µl TRITC sheep anti rabbit Ig for 30 minutes at 4 °C Between and after the incubations cells were washed with MEM and finally suspended in 50 µl MEM

The cells were examined immediately after incuba tion At least 300 cells were counted in a Leitz Orthoplan microscope equipped with Osram HBO 200 mercury lamp and the Ploemopak 2–1 vertical illuminator The filter combinations recommended by *Ploem* were used (17) together with Leitz oil objective 40/1 2 with periplan GW 6 3× eye pieces This method permitted simultaneous counting of FITC positive (B cells) and TRITC positive cells (T-cells) by changing filter combi nations

Immunofluorescence was always carried out with normal spleen and thymus cell suspensions as controls Prior to use the red cells in spleen and thymus suspensions were haemolysed with 0 14 M NH_4Cl for 10 minutes at room temperature By this method 93–97% of thymus cells were TRITC positive 38–42% of spleen cells were TRITC positive and 45–48% FITC positive Repeated experiments on seven identical samp

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morphological criteria in May Grunwald Giemsa stai ned smears and by the non specific esterase staining (24)

RESULTS

Cellular Composition of Peritoneal Exudate after a Single Intraperitoneal Injection of Glucan

The first set of experiments was carried out to determine the optimal dose of glucan for induction of cellular exudates in the peritoneal cavity Groups of 12 mice were injected intraperitoneally with 2 5 µg 5 µg 7 5 µg 10 µg 25 µg and 100 µg per mouse, and a control group was given PBS Two

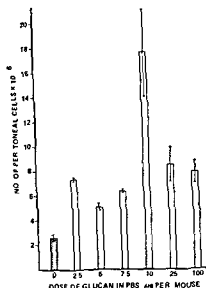


Fig 1 Total number of cells in peritoneal exudates 7 days after a single injection of various doses of glucan Mean and range of duplicates are stated

mice from each group were killed on days 2 4 7 14 21 and 35 after injection The peritoneal exudate cells were collected total cells were counted and the percentages of T-cells B-cells and macrophages were estimated as described

The total number of cells using various doses of glucan is illustrated in Fig 1 The optimal dose was found to be 10 µg/mouse (0 4 mg/kg) which gave 17 6 × 10^6 cells on day 7

The kinetics of the response after injection of 10 [illegible] shown in Fig 2 There was [illegible]

population increased about 3 fold after 7 to 14 days and was maintained for at least 35 days after inoculation of glucan

Effect of Injection of Glucan on Subsequent Tumour Take

The second set of experiments was performed to examine the effect of glucan on tumour take glucan being injected seven days before the inoculation of tumour, using the dose of glucan giving maximal effect on lymphocyte and macrophage influx A dose of 5 × 10^2 viable tumour cells was chosen thus achieving a tumour take of 80% in the control group

Two groups of 10 mice were given 10 µg glucan per mouse or PBS respectively seven days before tumour challenge Thereafter the animals were evaluated for tumour take twice weekly When such a low tumour dose was used the animals developed solid tumours which could be palpated when they reached a size of 3–4 mm The results are shown in Fig 3 Contrary to our expectations the glucan group eventually ended up with a slightly higher tumour take than the control group This difference is probably not significant

Number of Host Cells in Ascites tumours after Pre treatment with Glucan

The third set of experiments was performed to

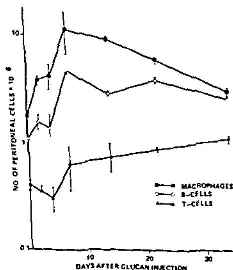


Fig 2 Peritoneal exudate content of T-cells B-cells and macrophages at various times after injection of 10 µg glucan per mouse Mean and range are stated The values at day 0 represent findings in untreated animals

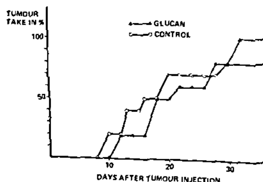


Fig 3 Effect on tumour take of pretreatment of hosts with glucan Two groups of 10 mice each were given 10 µg glucan per mouse or PBS respectively 7 days prior to tumour challenge with 5 × 10^2 viable tumour cells Tumour appearance was evaluated by palpation of the site of injection every 2nd to 3rd day All tumours grew progressively

AA subline grows in an ascites form when inoculated in doses higher than 10^5 viable tumour cells. With lower inoculation doses some animals develop a solid tumour at the site of the inoculum probably because of leakage of tumour cells after inoculation. The tumour is weakly immunogenic judged by the fact that inoculation of as few as 5×10^2 viable cells give progressive solid tumour growth in approximately 80% of the animals. The tumour was maintained in the ascites form by weekly transfer of 0.5–1 ml of ascites.

Glucan

A β1–3 yeast cell wall glucan was the gift of Professor *Jan Raa* University of Tromso Norway.

The glucan was suspended in phosphate buffered saline (PBS) in a concentration of 1 mg/ml and sonicated with a MSE sonicator for 45–60 seconds at medium power. The stock solution was sterilized by boiling and stored at 4 °C. If stored for more than a week the suspension was resonicated before use. Appropriate dilutions of the stock solution were made prior to injection so that the injected volume was constant.

Chemicals

The medium used for cell washing was Eagles minimum essential medium supplemented with Hepes buffer (MEM Hepes) obtained from Gibco Grand Island New York USA. Heparin was purchased from Nyegaard & Co Oslo Norway, rabbit anti mouse brain antibody from Cedarlane Ltd Wellington Rd S London Canada, rabbit immunoglobulin from Mann Research Laboratories Inc New York USA, tetramethyl rhodamine isothiocyanate (TRITC) from Baltimore Biological Lab Cockeysville Maryland USA, fluorescein isothiocyanate (FITC) from Koch Light Lab Colnbrook Buckinghamshire England, human IgG from Kabi Stockholm Sweden, sepharose from Pharmacia Fine Chemicals Uppsala Sweden and staining solutions for May Grunwald Giemsa staining from Merck Darmstadt West Germany. For nonspecific esterase staining basic fuchsin was obtained from Koch Light Lab, α naphtyl acetate from Sigma Chemical Co St Louis Maryland USA and methyl green from Difco Lab Surrey England.

Antibodies

The FITC F(ab')$_2$ goat anti mouse Ig was produced purified and conjugated with fluorochromes as described by *Hannestad & Gaudernack* (9). Sheep anti rabbit Ig was produced by immunization of sheep with rabbit Ig precipitated with 50% saturated ammonium sulphate purified by binding to and elution from rabbit IgG linked to sepharose and conjugated with TRITC as described by *Bergquist & Nilsson* (1). All antibodies were inactivated at 56 °C for 30 minutes and diluted in MEM containing 10 mg/ml human IgG aggregated at 63 °C for 10 minutes to block the Fc receptors of the cells. The optimal dilutions of each antibody were titrated separately.

Collection of Cells

Animals were killed by overdoses of ether and peritoneal exudate cells (PEC) or ascites tumour suspensions were collected by aspiration following the injection of 2 ml phosphate buffered saline with heparin (15 IU/ml). Cells were counted in a Burker haemacytometer. All cell suspensions were washed with MEM Hepes. Viability of cells was tested by trypan blue exclusion. The viability of PEC was usually higher than 95%. The tumour cell viability decreased to about 85% within a few hours of collection but then remained constant throughout the immunofluorescence procedure.

Identification of Cells

Theta positive cells (T-cells) and surface Ig positive cells (B-cells) were identified by immunofluorescence as follows. Suspensions of PEC or ascites cells from tumour bearing mice were collected and washed twice with MEM hepes. Tumour cell suspensions which were visibly bloody were excluded from the experiments. Cells were counted and 10^6 cells were incubated at 4 °C for 60 minutes with 50 µl rabbit anti mouse brain antibody. They were then incubated with 50 µl FITC F(ab')$_2$ goat anti mouse Ig for 30 minutes at 4 °C. Finally the cells were incubated with 50 µl TRITC sheep-anti rabbit Ig for 30 minutes at 4 °C. Between and after the incubations cells were washed with MEM and finally suspended in 50 µl MEM.

The cells were examined immediately after incubation. At least 300 cells were counted in a Leitz Orthoplan microscope equipped with Osram HBO 200 mercury lamp and the Ploemopak 2–1 vertical illuminator. The filter combinations recommended by *Ploem* were used (17) together with Leitz oil objective 40/1.2 with periplan GW 6.3× eye pieces. This method permitted simultaneous counting of FITC positive (B-cells) and TRITC positive cells (T-cells) by changing filter combinations.

Immunofluorescence was always carried out with normal spleen and thymus cell suspensions as controls. Prior to use the red cells in spleen and thymus suspensions were haemolysed with 0.14 M NH_4Cl for 10 minutes at room temperature. By this method 93–97% of thymus cells were TRITC positive, 38–42% of spleen cells were TRITC positive and 45–48% FITC positive. Repeated experiments on seven identical samples of a spleen cell suspension gave a variation in range of B cells of 46 ± 2% (mean ± S.D.) and T cells of 40 ± 2% (mean ± S.D.). Macrophages were identified by morphological criteria in May Grunwald Giemsa stained smears and by the non specific esterase staining (24).

RESULTS

Cellular Composition of Peritoneal Exudate after a Single Intraperitoneal Injection of Glucan

The first set of experiments was carried out to determine the optimal dose of glucan for induction of cellular exudates in the peritoneal cavity. Groups of 12 mice were injected intraperitoneally with 2.5 µg, 5 µg, 7.5 µg, 10 µg, 25 µg and 100 µg per mouse and a control group was given PBS. Two

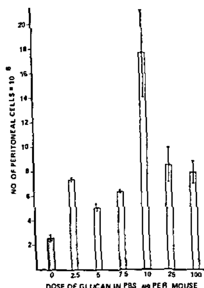


Fig 1 Total number of cells in peritoneal exudates 7 days after a single injection of various doses of glucan Mean and range of duplicates are stated

mice from each group were killed on days 2 4, 7 14 21 and 35 after injection The peritoneal exudate cells were collected total cells were counted and the percentages of T-cells B-cells and macrophages were estimated as described

The total number of cells using various doses of glucan is illustrated in Fig 1 The optimal dose was found to be 10 µg/mouse (0 4 mg/kg) which gave $17\,6 \times 10^6$ cells on day 7

The kinetics of the response after injection of 10 µg glucan per mouse is shown in Fig 2 There was an immediate increase in macrophages and B-cells, both reaching a maximum at day 7 The T-cell population increased about 3 fold after 7 to 14 days and was maintained for at least 35 days after inoculation of glucan

Effect of Injection of Glucan on Subsequent Tumour Take

The second set of experiments was performed to examine the effect of glucan on tumour take, glucan being injected seven days before the inoculation of tumour using the dose of glucan giving maximal effect on lymphocyte and macrophage influx A dose of 5×10^2 viable tumour cells was chosen, thus achieving a tumour take of 80% in the control group

Two groups of 10 mice were given 10 µg glucan per mouse or PBS respectively, seven days before tumour challenge Thereafter the animals were evaluated for tumour take twice weekly When such a low tumour dose was used, the animals developed solid tumours which could be palpated when they reached a size of 3–4 mm The results are shown in Fig 3 Contrary to our expectations, the glucan group eventually ended up with a slightly higher tumour take than the control group This difference is probably not significant

Number of Host Cells in Ascites tumours after Pretreatment with Glucan

The third set of experiments was performed to

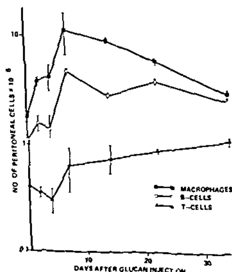


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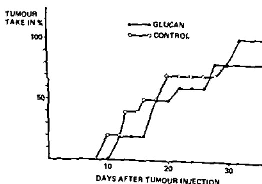


Fig 3 Effect on t [illegible] appearance was evaluated by palpation of the site of injection every 2nd to 3rd day All tumours grew progressively

find out whether pretreatment with glucan altered the content of T-cells, B cells and macrophages in ascites tumours Two groups of 20 mice each were injected intraperitoneally with 10 µg glucan per mouse or an equal volume of PBS One million viable tumour cells were injected, at which dose an ascites tumour develops The advantage of using doses that produce an ascites tumour in this type of experiment is obvious, though the disadvantage is that a high tumour dose does not give the immune apparatus a »fair« chance to control tumour growth With this tumour dose, the mice died approximately 18–20 days after injection At day 3, 7, 12 and 16 after tumour inoculation, five mice from each group were killed and the ascites collected

The total number of each cell type on various days after tumour challenge is shown i Fig 4 There was an initial small increase in the number of macrophages and B-cells in the glucan group, but the numbers decreased to the level of the control group at a later stage T-cell content showed the opposite development Initially there was no significant difference between the two groups, but later the content of T-cells in the glucan group increased more than in the control group

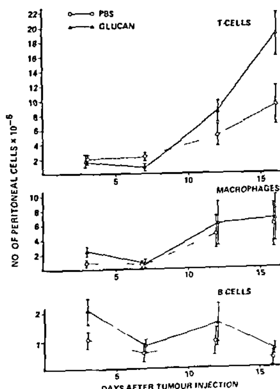


Fig 4 Effect f pretreatment with glucan on the total number of T cells B cells and macrophages in a murine ascites tumour Two groups of 20 mice were injected with 10 µg glucan per mouse or PBS respectively 7 days prior to challenge with 10^6 viable tumour cells On day 3, 7 12 and 16 after tumour injection five mice from each group were killed and the total numbers of intratumoural T cells B cells and macrophages were estimated using immunofluorescence and non specific esterase staining Mean and range are stated for each point of time

DISCUSSION

The antitumour effect of glucan has been demonstrated in many different experimental tumour systems, but the exact mechanism behind this effect is not known It is commonly supposed that the macrophage is the effector cell responsible for this effect (2, 6), and there is some evidence in favour of this view Glucan has a strong stimulative effect on macrophages *in vitro* (5) Cytostatic macrophages have been isolated from regressing tumours treated with glucan (2, 14), and transfer of the antitumour effect can be obtained with peritoneal cells but not with lymph node cells or serum (14) Morphologically, a prominent macrophage infiltration is observed in solid tumours after glucan treatment (5 15)

The *in vivo* antitumour effect of glucan also depends on an intact T-cell function (12, 13) and stimulatory effects of glucan on various T-cell functions have been reported (4, 7, 8)

Our experiments demonstrate an increase in macrophages, T-cells and B-cells after a single intraperitoneal injection of glucan The increase in macrophage numbers was greatest while the increase in T-cells lasted longer Similar findings have been demonstrated with another antitumour agent, pyran (23) *Stylos et al* demonstrated that administration of pyran resulted in an increase of T-cells and a temporary increase in B-cell population in spleens of tumour-bearing mice as compared to tumour-bearing controls However, we were not able to demonstrate any antitumour effect of glucan, although the tumours were injected when the number of macrophages in peritoneal exudate had increased 8 fold following glucan pretreatment

There are several possible explanations for the lack of antitumour effect of glucan in our experiments despite the striking effect on cell numbers One reason – though quite improbable – is that the optimal antitumour dose may not correspond to the dose that gives the highest cell influx Another is that this special tumour exerts an anti macrophage or anti T-cell effect that overwhelms the tumouricidal capacity of the host cells Actually, a depressive effect of tumour products on macrophage functions has been described (16, 21, 22) A third possibility no less probable than the other two, is that the glucan particles might actually divert the immune

cells from contact with the tumour cells Thus an antitumour effect might not only be dependent on the dose of glucan but also on the application of the compound into the tumour

In order to elucidate which of these possibilities is the correct one much more must be known concerning the effects of the glucan on macrophages and lymphocytes The fact that in some systems glucan gives a dramatic regression of tumours and under other circumstances has no effect at all necessitates more detailed studies on the mechanism of action of this and similar drugs

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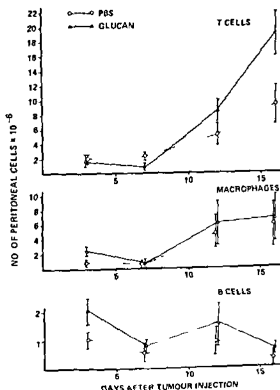


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FURTHER CHARACTERIZATION OF HUMAN SUPPRESSOR CELLS GENERATED IN MIXED LYMPHOCYTE CULTURES

ERIK ARNESEN and ERIK THORSBY

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Arnesen E & Thorsby E Further characterization of human suppressor cells generated in mixed lymphocyte cultures Acta path microbiol scand Sect C 88 103-108 1980

Suppressor cells can be generated in mixed lymphocyte cultures (MLC) The studies reported here show that the cells responsible for suppression belong to relatively radioresistant T cells Furthermore to generate suppressor cells in MLC the cells must be able to proliferate while this is not necessary for expression of the suppressive capacity

Key words Suppressor cells mixed lymphocyte culture

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Suppression of immune phenomena by lymphoid cells both *in vivo* and *in vitro* is well established (3) Different sub populations have been shown to have a suppressive or regulatory function in animals (3 15) *In vitro* activation of human suppressor cells in cultures of peripheral blood mononuclear cells (PBM) or spleen cells has been demonstrated by stimulation with Concanavalin A (Con A) (18 19 10 17 2) and phytohaemaglutinin (PHA) (18 2 11) as well as by allogeneic cells in mixed lymphocyte cultures (MLC) (8) Cells which suppress MLC interactions have also been found to be present in certain individuals *in vivo* and studies by *McMichael & Sasazuki* (13) and *Engleman et al* (4) have shown that they appear to belong to a population of relatively radioresistant T cells The suppressor cells which can be generated in *in vitro* MLC tests are however less well characterized

The purpose of these investigations was to further characterize the suppressor cells which can be generated in *in vitro* MLC tests

MATERIALS AND METHODS

Cell donors Healthy persons from the laboratory staff were used as blood donors PBM cells were prepared by Ficoll Isopaque flotation (»Lymphoprep« Nyegaard & Co Oslo Norway)

T and non T cells were prepared by means of sheep-erythrocyte (E) rosetting as previously described from this laboratory (1) Briefly 1×10^7 PBM cells in 5 ml medium (RPMI 1640 Gibco Biocult, Glasgow Scotland with 100 IU/ml penicillin and 100 μg/ml streptomycin added) were mixed at 20 °C with 5 ml medium containing 1% AET (2-aminoethylisothiouronium bromide hydrobromide Sigma St Louis Mo USA) treated sheep red blood cells (SRBC) and 40% foetal bovine serum (Gibco Biocult) in 50 ml plastic tubes (no 2070 Falcon Oxnard Cal USA) and centrifuged at 200 *g* at 20 °C for 5 min The sediment was then gently resuspended using a wide bore pipette The suspension was layered over 10 ml Lymphoprep in a 50 ml tube and centrifuged at 800 *g* for 20 min at 20 °C The interphase containing non E rosetting cells was removed resuspended in medium and used as non T cells These suspensions usually contained 60-90% Ig positive cells and 20-30% latex ingesting cells The residual supernatant was aspirated and the pellet of E rosetting cells was mixed with 2 ml autologous serum diluted 1 2 in medium and incubated for 15 min at 37 °C causing lysis of the SRBC (by xeno-antibodies in human serum) The remaining cells were washed twice resuspended in medium containing 20% normal human serum and used as T cell enriched suspensions They usually contained 70% E rosette forming cells and less than 5% Ig positive and 5% latex ingesting cells

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Arnesen E & Thorsby E Further characterization of human suppressor cells generated in mixed lymphocyte cultures Acta path microbiol scand Sect C 88 103–108 1980

Suppressor cells can be generated in mixed lymphocyte cultures (MLC) The studies reported here show that the cells responsible for suppression belong to relatively radioresistant T cells Furthermore to generate suppressor cells in MLC the cells must be able to proliferate while this is not necessary for expression of the suppressive capacity

Key words Suppressor cells mixed lymphocyte culture

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Suppression of immune phenomena by lymphoid cells both *in vivo* and *in vitro* is well established (3) Different sub-populations have been shown to have a suppressive or regulatory function in animals (3 15) *In vitro* activation of human suppressor cells in cultures of peripheral blood mononuclear cells (PBM) or spleen cells has been demonstrated by

lymphocyte cultures (MLC) (8) Cells which suppress MLC interactions have also been found to be present in certain individuals *in vivo* and studies by *McMichael & Sasazuki* (13) and *Engleman et al* (4) have shown that they appear to belong to a population of relatively radioresistant T cells The suppressor cells which can be generated in *in vitro* MLC tests are however less well characterized

The purpose of these investigations was to further characterize the suppressor cells which can be generated in *in vitro* MLC tests

MATERIALS AND METHODS

Cell donors Healthy persons from the laboratory staff were used as blood donors PBM cells were prepared by Ficoll Isopaque flotation (»Lymphoprep« Nyegaard & Co Oslo Norway)

T and non T cells were prepared by means of sheep-erythrocyte (E) rosetting as previously described from this laboratory (1) Briefly 1×10^7 PBM cells in 5 ml medium (RPMI 1640 Gibco Biocult, Glasgow Scotland with 100 IU/ml penicillin and 100 µg/ml streptomycin added) were mixed at 20 °C with 5 ml medium containing 1% AET (2 aminoethylisothiouronium bromide hydrobromide Sigma St Louis Mo USA) treated sheep red blood cells (SRBC) and 40% foetal bovine serum (Gibco Biocult) in 50 ml plastic tubes (no 2070 Falcon Oxnard Cal USA) and centrifuged at 200 *g* at 20 °C for 5 min The sediment was then gently resuspended using a wide bore pipette The suspension was layered over 10 ml Lymphoprep in a 50 ml tube and centrifuged at 800 g for 20 min at 20 °C The interphase containing non E rosetting cells was removed resuspended in medium and used as non T cells These suspensions usually contained 60–90% Ig positive cells and 20–30% latex ingesting cells The residual supernatant was aspirated and the pellet of E rosetting cells was mixed with 2 ml autologous serum diluted 1 2 in medium and incubated for 15 min at 37 °C causing lysis of the SRBC (by xeno-antibodies in human serum) The remaining cells were washed twice resuspended in medium containing 20% normal human serum and used as T cell enriched suspensions They usually contained 70% E rosette forming cells and less than 5% Ig positive and 5% latex ingesting cells

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FURTHER CHARACTERIZATION OF HUMAN SUPPRESSOR CELLS GENERATED IN MIXED LYMPHOCYTE CULTURES

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Suppression of immune phenomena by lymphoid cells both *in vivo* and *in vitro* is well established (3) Different sub-populations have been shown to have a suppressive or regulatory function in animals (3 15) *In vitro* activation of human suppressor cells in cultures of peripheral blood mononuclear cells (PBM) or spleen cells has been demonstrated by stimulation with Concanavalin A (Con A) (18 19 10 17 2) and phytohaemaglutinin (PHA) (18 2 11) as well as by allogeneic cells in mixed lymphocyte cultures (MLC) (8) Cells which suppress MLC interactions have also been found to be present in certain individuals *in vivo* and studies by *McMichael & Sasazuki* (13) and *Engleman et al* (4) have shown that they appear to belong to a population of relatively radioresistant T cells The suppressor cells which can be generated in *in vitro* MLC tests are however less well characterized

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TABLE 2 *Effect of Different »Bulk« MLC culturing Times*

»Bulk« MLC[a] culturing time in days	Experiment 1: AB_x[b]	Experiment 1: AB_x + »Bulk« (AB_x) 800 rads	Experiment 1: % Δ[c]	Experiment 2: AB_x[b]	Experiment 2: AB_x »Bulk« (AB_x) 800 rads	Experiment 2: % Δ[c]
2	25718	20183	−21	12677	3836	−69
4	26068	19601	−24	15902	3837	−75
6	18218	2512	−86	14146	2688	−81
8	21905	3977	−82	26061	5326	−79
10	8041	2724	−66	14126	4545	−68
14	19375	8225	−58	18479	3485	−81
21	31780	21980	−31	28651	38120	+ 33

(Column heading: ^3H thymidine incorporation (cpm) in response to allogeneic stimulating cells — Test cultures)

a) »Bulk« MLC activated cells given 800 rads before addition to test MLC culture
b) Freshly established micro MLC cultures (5×10^4 responder and stimulator cells)
c) % inhibition

[illegible]

cytotoxic T cells generated in the primary »bulk« MLC directed towards the foreign HLA antigens on the stimulating cells However no differences in the inhibitory effect could be detected

Kinetics and Dose response Investigations

»Bulk« MLC were cultured for two to 21 days given 800 rads and added to freshly established test MLCs Table 2 shows the results of two experiments Pronounced suppressor activity is present in the »bulk« cultures after 6–14 days of culture At day 21 of culture the inhibition was less pronounced or had disappeared However the »bulk« cultures were still viable since it was possible to induce new proliferation of the »bulk« culture cells after 21 days of culture when confronting them with freshly prepared unrelated irradiated stimulating cells (data not shown)

The number of MLC-activated T cells necessary to observe suppression was also studied Table 3 shows one typical experiment out of four Suppression disappeared when less than 2.5 × 10^4 MLC activated cells were added to 5 × 10^4 freshly prepared responding and stimulating cells In fact

TABLE 3 *Effect of Addition of Different Concentrations of Mixed Lymphocyte (MLC)-activated Lymphocytes to Freshly Established Micro MLC*

Freshly established test MLC culture[a]	0	5×10^4	2.5×10^4	1×10^4	0.5×10^4	0.25×10^4	0.2×10^4	0.1×10^4
	Number of »bulk« (AB_x) cells added[b] without previous irradiation							
AA_x	1984	5036	5243	3223	5650	3903	3809	3474
AB_x	19209	7019	14359	18500	27767	34039	38615	44410
	Irradiated with 800 rads before addition							
AA_x		2466	2021	1152	1679	1810	811	1579
AB_x		9828	12483	16211	21129	16766	15923	19765

(Column heading: ^3H thymidine incorporation (cpm) in response to autologous or allogeneic stimulating cells)

a 5×10^4 responding and stimulating cells
b T-enriched lymphocytes activated for 6 days in »bulk« MLC

Generation of suppressor cells (»bulk« MLCs) MLCs were established as previously described (8) Briefly 2.5 × 10⁶ PBM cells or suspensions enriched for T or non T cells were incubated with 5.0 × 10⁶ X ray irradiated (2000 rads subscript$_x$) stimulating autologous or allogeneic mononuclear cells in 15 ml culture medium (RPMI 1640 supplemented with 20% inactivated normal human serum and antibiotics) in 30 ml plastic flasks (Falcon Plastics Cat no 3012) Incubation was carried out at 37 °C in a humidified atmosphere of air and 5% CO_2 After a given culture time (usually six days) the cells were washed once and resuspended in culture medium

Test MLC cultures One way micro MLCs were established with fresh PBM from the original and third party blood donors usually six days after initiation of the »bulk« MLC Equal numbers (5 × 10⁴) of responding and X ray irradiated stimulating cells were mixed in the wells of microtitre plates (C A Greiner und Söhne Nürtingen West Germany) in a volume of 0.10 ml culture medium To test their suppressive activity either 0.05 ml culture medium or 0.05 ml (5 × 10⁴) cells from the »bulk« MLCs were added The MLC activated cells were used either as not enriched or T enriched cells and given a variable dose of X ray irradiation (0 400 800 1200 1600 2000 3000 or 5000 rads) by varying the irradiation time from 3½ min while keeping the voltage (250 kV) and ampere (12mA) the same The microtitre plates were incubated for an additional 5 days labelled 24 hours before harvesting with ³H thymidine (2.0 µCi/mMol New England Nuclear Boston Mass USA) and harvested on a Skatron semi automatic harvesting machine (Skatron Lierbyen Norway) the results were expressed as mean cpm of triplicate cultures ∓ standard error of the mean However the latter value is not expressed in the tables since it was almost always less than 10% The results were also expressed as percentage suppression (% Δ) from the formula

$$\% = \frac{\text{(Mean cpm suppressor cells added}}{\text{Mean cpm control culture}} \times 100) - 100$$

RESULTS

Cells Responsible for Suppression

Whether T cells were responsible for the suppression was studied first Table 1 shows the results of one out of four experiments giving similar results An equal inhibition was caused by PBM and T enriched cells (A_TB_x) when the enrichment was performed before establishing the »bulk« MLC Only small inhibition was caused when non T cells were used as responding cells ($A_{non\,T}B_x$) When separating into T and non T cells after six days of culture only very few non T cells could be recovered On the other hand Table 1 shows that both enriched T cells (($AB_x)_T$) and unfractionated cells (AB_x) from six day MLC cultures exerted the same degree of inhibition There was usually no clear specificity of the inhibition i e a similar degree of inhibition was seen whether the same (AB_x) or third party (AC_x) stimulating cells were used in the test cultures

In order to study whether proliferation was necessary for the activation of suppressor cells X ray irradiated (2000 rads) cells from two individuals A and B were cultured together for six days and added to freshly established AA_x and AB_x cultures (Table 1) No inhibition was seen

TABLE 1 *Effect of Addition of Mixed Lymphocyte Culture (MLC)-activated Lymphocytes to Freshly Established Micro MLC either Unseparated or Separated into T and Non T Cells before or after 6 Days of Activation*

MLC culture cells added[a]	³H thymidine incorporation (cpm) in test cultures				
	AAx	ABx	% Δ	ACx	% Δ
–	221	10702		13327	
(ABx)	647	3519	−67	6873	−48
(A_TBx)[b]	44	3375	−68	7864	−41
($A_{non\,T}$Bx)[b]	1038	8613	−20	11978	10
–	3035	12501		19784	
(ABx)	4301	2910	−77	16743	15
$(ABx)_T$[c]	5412	2237	−82	10253	−48
(AxBx)	11828	16265	+ 30	18103	− 8

a) 5 × 10⁴ cells from these 6 day cultures were added to an equal number of autologous responding and allogeneic stimulating cells

b) Separation of T and non T cells from donor A before activation

c) Separation of T cells from the allogeneic mixture after 6-day MLC activation

TABLE 2 *Effect of Different »Bulk« MLC-culturing Times*

»Bulk« MLC[a] culturing time in days	^{3}H thymidine incorporation (cpm) in response to allogeneic stimulating cells Test cultures					
	Experiment 1			*Experiment 2*		
	AB_x[b]	AB_x + »Bulk« (AB_x) 800 rads	% Δ[c]	AB_x[b]	AB_x »Bulk« (AB_x) 800 rads	% Δ[c]
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a) »Bulk« MLC activated cells given 800 rads before addition to test MLC culture
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In some experiments ...

done in order to facilitate detection of possible cytotoxic T cells generated in the primary »bulk« MLC, directed towards the foreign HLA antigens on the stimulating cells However, *no differences in* the inhibitory effect could be detected

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Freshly established test MLC culture[a]	^{3}H thymidine incorporation (cpm) in response to autologous or allogeneic stimulating cells							
	Number of »bulk« (AB_x) cells added[b] without previous irradiation							
	0	5×10^4	2.5×10^4	1×10^4	0.5×10^4	0.25×10^4	0.2×10^4	0.1×10^4
AA_x	1984	5036	5243	3223	5650	3903	3809	3474
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b T-enriched lymphocytes activated for 6 days in »bulk« MLC

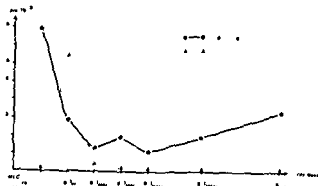


Fig 1a Effect of different doses of X ray *inactivation* of allogeneic 6 day »bulk« MLC culture cells (AB_x) before addition to freshly established test MLC culture AB_x ●——● The »bulk« MLC *activated cells confronted* with irradiated (2000 rads) third party *cells* C_x ▲ ▲ demonstrate at which dose the proliferative capacity of the activated cells (AB_x) disappeared

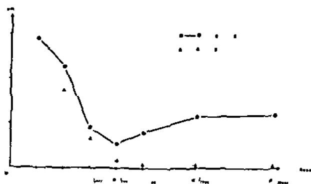


Fig 1b Effect of different doses of X ray irradiation of allogeneic 6 day bulk MLC culture cells (AB_x) before addition to freshly established test MLC culture AB_x ●——● The bulk MLC activated cells confronted with irradiated (2000 rads) third party cells C_x ▲ ▲ demonstrate at which dose the proliferative capacity of the activated cells (AB_x) disappeared

when smaller numbers of cells were added potentiation was observed if the MLC activated cells had not been pre irradiated with 800 rads After irradiation no potentiation was seen

X ray Sensitivity of the Suppressing Cells

The MLC activated cells were treated with X ray irradiation doses from 400 to 5000 rads before addition to the test MLCs Figures 1a and b show the results of two typical experiments out of seventeen Figure 1a shows that suppression was still present when the added »bulk« cells had first been given 400 3000 rads In other experiments we could demonstrate no or only little suppression if irradiation of the added cells was not carried out while pronounced inhibition was always found after 400 or 800 rads of irradiation (Fig 1b) Even when treated with 5000 rads this inhibiting effect was not completely abrogated Thus the suppressing cells are relatively radioresistant This radioresistance was highly reproducible as witnessed in experiments where suppressor cells were generated from a single donor on five separate occasions (data not shown)

In some experiments »bulk« cells which had first been killed by heating (56 °C) were added to test cultures These killed cells had no inhibitory effect on the test cultures

To compare the effect of X ray irradiation on the proliferative and the suppressive capacity the added cells were also stimulated with third party X ray irradiated (2000 rads) cells (C_x) As shown in the figures the proliferative capacity almost disappeared when the MLC activated cells were first treated with 800 rads or more Restimulation with freshly prepared irradiated cells from B gave the same result (not shown in the figures)

DISCUSSION

It has previously been reported from this laboratory that non specific suppressor cells can be activated in MLC (8) The main findings of the experiments reported here are that the suppressing cells are most probably relatively radioresistant T cells Further to generate suppressor cells the cells must be able to proliferate while this is not necessary for expression of their suppressing activity

Previous studies have shown that maximum inhibition was found on day 5 of the test cultures and that the detectable suppression was not due to some changes in the kinetics of the test MLCs *(8)* For this reason in the present study harvesting of test culture was carried out only on day 5

T cells were found to be necessary for the generation of potent suppression and the degree of suppression was similar with activated T enriched and PBM cells (Table 1) These findings are in agreement with those previously found in mice (9 16) and *in vivo* in man (13 4) After our studies were finished *Hanes et al* (7) reported that also in their MLC experiments the suppressing cells were found among the T cells These suppressor T cells may express HLA DR antigens (8)

We found that pronounced suppression was always caused by six day MLCs while a more variable suppression was seen in MLCs cultured for a shorter time (Table 2) *Miller et al* (14) have reported that when culturing MLCs for three six and nine days significant suppression was found only after nine days of culture On the other hand in some experiments pronounced suppression was

also caused by 2–4 day MLCs (Table 2) at a time when little proliferation is seen in primary MLCs This may be compared with observations of *Sakane & Green* (17) who found that proliferation was not necessary for Con A to induce suppressor cells However when the cells were inhibited by X ray irradiation to proliferate at all no suppressor cells were generated (Table 1)

The dose response experiments demonstrated that for suppression to be observed the ratio between the added suppressing T cells and the freshly prepared responding cells had to be at least 1 2 (Table 3) Potentiation was observed when fewer cells were added The reason for the potentiation is unknown but may indicate that after six days of MLC a mixture of primed MLC responding T cells and suppressor T cells exists and that the latter can only be detected when more than a certain minimum number of cells are added to the test cultures The lack of potentiation seen when the proliferative capacity of the added cells were first inhib ted by giving 800 rads supports this sugges tion

The experiments reported here revealed that the suppressive effect was relatively resistant to X ray irradiation and more resistant than the ability to prol ferate when confronted with allogeneic cells This is in agreemen [illegible] found total elimination of suppression with doses of 500 rads or more in CMC with mouse spleen cells In man *Hanes et al* (7) found that the suppressor cells were sens tive to 3000 rads In this study suppressor activity remained intact after exposure to up to 800 rads wh le less [illegible] r po ed previously from our laboratory namely that MLC generated suppressor cells are sensitive to 2000 rads of irradiation (8) However in our previous study the radiosensitivity was tested only with 2000 rads of irradiation and in some experiments only a small reduction in suppressive activity was found The rather extreme radioresis tance found in the present studies raises the question as to whether these suppressor cells have to be viable at all in order to suppress However killed cells had no suppressive effect

The results reported here also suggest that a poss ble secondary proliferative wave of the »bulk MLC-activated cells when confronted with new stimulating cells may overshadow the ability to suppress [illegible] MLCs and may explain why no suppression was demonstrated in some of the experiments

An important question is whether the suppres sion at least in part is due to generation of cytotoxic T cells during the initial »bulk« MLC killing the freshly added stimulating cells *Hirschberg & Thorsby* (8) found this to be unlikely in experiments with primary and secondary stimula ting cell combinations selected to share as few HLA A and B antigens as possible In the experiments reported here the potential effector to target cell ratio was usually kept at 1 1 and even when the stimulator cell concentration was increased two and [illegible] but do not establish firmly that suppressor and not cytotoxic T cells are mostly responsible for the inhibition That suppressor and cytotoxic cells belong at least in part to different subsets of T cells is further suggested by other recent studies in our laboratory Generation of suppressor cells seems to be more resistant to the effects of low doses of steroids than generation of cytotoxic cells (*Hirschberg* personal communication)

Evidence has been presented that suppressor cells may be involved in transplantation tolerance (for references see (20)) It would therefore be of interest to study whether the lymphocytes of recipients of well functioning grafts have suppressor activity Some recent reports indicate that this is the case (6 12 21 22) However further studies are needed particularly to obtain better insight into the possible specificity of these suppressor T cells

The authors wish to express their gratitude to Dr *Henry Hirschberg* for helpful suggestions during this work, and to *Helena Nous a nen* for skilful technical assistance This work was supported by grants from the *Norwegian Society for Sc ence and the Human es* and *Mr Anders Jahre s Med cal Research Fund*

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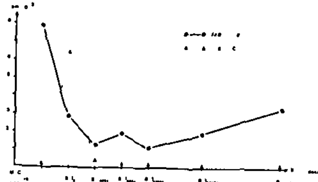


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DEGRADATION OF NON-OPSONIZED ^{32}P-LABELLED *E COLI* BY UNSTIMULATED MOUSE PERITONEAL MACROPHAGES

Evaluation of a Method

HALVOR ROLLAG

Kaptein W Wilhelmsen og Frues Bakteriologiske Institutt University of Oslo National Hospital of Norway Oslo Norway

Rollag H Degradation of non opsonized ^{32}P labelled *E coli* by unstimulated mouse peritoneal macrophages Evaluation of a method Acta path microbiol scand Sect C 88 109–114 1980

Five hours after the ingestion of non opsonized *E coli* by mouse peritoneal macrophages *in vitro* 60–70 per cent of the bacteria were killed estimated as decrease in colony forming units When employing ^{32}P labelled *E coli* 50–60 per cent of the radioactivity was released to the medium by the macrophages during the same period of time The mean number of visually-counted bacteria per macrophage was constant in the post ingestive period The macrophages killed the bacteria without early destruction The release of radioactivity seemed to reflect the degradation of bacteria in the macrophages

Key words Macrophages phagocytosis *E coli*

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One of the most critical factors in the study of interaction between microorganisms and phagocytic cells is the measurement of microbial death Several techniques have been employed for *in vitro* study of the microbicidal activity of phagocytic cells The method most frequently employed is probably the assessment of microbial survival by determination of colony forming units (CFU) The extensive studies of *Cohn* (2) however led to increasing employment of radioactive labelled bacteria and to the measuring of the release of label from the prelabelled bacteria as a means of quantifying killing

The fate of non-opsonized bacteria in phagocytes has been disputed since *Rowley* (12) reported that previous opsonization was necessary for the killing of *E coli* phagocytized by mouse peritoneal macrophages (MPM)

The aim of the present work is to evaluate how measuring the radioactivity released by macrophages after ingestion of ^{32}P labelled non-opsonized *E coli* may be used as a method for studying the fate of this bacterium after phagocytosis

MATERIALS AND METHODS

Bacteria

E coli strain X 7 serogroup O 86 B 7 as utilized in previous experiments was used (11) Preparation of the bacterial suspension and labelling of the bacteria with ^{32}P-orthophosphate was performed as described by *Midtvedt & Melby* (9) The suspending medium was Krebs Ringer phosphate buffer with 10 mM glucose (KRG) When not otherwise stated the experiments were performed using a bacterial suspension of $10^9 \pm 0.5$ CFU per ml

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DEGRADATION OF NON-OPSONIZED ^{32}P-LABELLED *E COLI* BY UNSTIMULATED MOUSE PERITONEAL MACROPHAGES

Evaluation of a Method

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Rollag H Degradation of non-opsonized ^{32}P labelled *E coli* by unstimulated *mouse peritoneal* macrophages Evaluation of a method Acta path microbiol scand Sect C 88 109–114 1980

Five hours after the ingestion of non-opsonized *E coli* by mouse peritoneal macrophages *in vitro* 60–70 per cent of the bacteria were killed estimated as decrease in colony forming units *When employing* ^{32}P labelled *E coli* 50–60 per cent of the radioactivity was released to the *medium by the* macrophages during the same period of time The mean number of visually-counted *bacteria per* macrophage was constant in the post ingestive period The macrophages *killed the* bacteria without *early destruction The release* of radioactivity seemed to reflect the degradation of bacteria in the macrophages

Key words Macrophages phagocytosis *E coli*

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One of the most critical factors in the study *of* interaction between microorganisms and *phagocytic* cells is the measurement of microbial death Several techniques have been employed for *in vitro* study of the microbicidal activity of phagocytic cells *The* method most frequently employed is probably the assessment of microbial survival by determination *of* colony forming units (CFU) The extensive studies of *Cohn* (2) however led to increasing employment of radioactive labelled bacteria and to the measuring of the release of label from the prelabelled bacteria as a means of quantifying killing

The fate of non-opsonized bacteria in phagocytes has been disputed since *Rowley* (12) reported that previous opsonization was necessary for the killing of *E coli* phagocytized by mouse peritoneal

The aim of the present work is to evaluate how *measuring the* radioactivity released by macrophages after ingestion of ^{32}P labelled non-opsonized *E coli* may be used as a method for studying the fate of this bacterium after phagocytosis

MATERIALS AND METHODS

Bacteria

E coli strain X 7 serogroup O 86 B 7 as utilized in previous *experiments* was *used (11)* Preparation of the bacterial *suspension and labelling* of the bacteria with ^{32}P-orthophosphate was *performed* as described by *Midtvedt & Melby* (9) The suspending medium was Krebs Ringer phosphate buffer with 10 mM glucose (KRG) When not otherwise stated the experiments were performed using a bacterial suspension of $10^{9 \pm 0.5}$ CFU per ml

Mouse Peritoneal Macrophages

Mouse peritoneal macrophages (MPM) were obtained from unstimulated male Ham/ICR/CSE/Bom albino mice Harvesting and processing of MPM were performed as described in a previous paper (11) MPM were cultivated on glass coverslips in Leighton tubes Eagle s Hanks minimal essential medium (Grand Island Laboratories New York) supplemented with 0 125 per cent $NaHCO_3$ and 20 per cent foetal bovine serum served as culture medium Except for 5 μg/ml of oxytetracycline (Dumex Copenhagen) in the harvesting medium no medium contained any antibiotic

Phagocytosis

The kinetics of phagocytosis of non opsonized *E coli* by MPM has been described earlier (11) MPM on glass coverslips in Leighton tubes were incubated overnight washed in KRG and and transferred to new Leighton tubes containing the radioactive labelled bacteria in suspension The tubes were incubated at 37 °C in 5 per cent CO_2 atmosphere for 30 min At this point coverslips were removed washed thoroughly in KRG and subsequently put into new Leighton tubes with fresh KRG The tubes were incubated for 20 more min to complete the ingestion of bacteria

Release of ³²P from the Bacteria to the Medium by MPM

At the end of the ingestion period coverslips were removed and cautiously washed in KRG to eliminate glass adherent and non ingested bacteria The coverslips were then transferred to new Leighton tubes with fresh KRG and further incubated At pre set intervals three parallels were tested for the distribution of the ^{32}P content The tubes were agitated the coverslips removed and the medium put on ice The coverslips were transferred to tubes containing 1 2 ml 1N NaOH In this alkaline solution the macrophages were detached and dissolved Protein content and radioactivity of the dissolved macrophages were determined as previously described (11) The macrophage associated radioactivity was expressed as counts per mg cell protein and min (CPMM)

From the medium saved on ice aliquots of 100 μl were removed for ^{32}P determination The remainder of the medium was centrifuged at 10000xg and 2 °C for 10 minutes to pellet radioactivity bound to particles Release of soluble ^{32}P from the bacteria into the medium by MPM could be expressed either as decrease in macrophage bound radioactivity or as increase in soluble ^{32}P in the medium The increase in soluble ^{32}P can be calculated from the quotient

$$\frac{^{32}P \text{ in the centrifuged medium}}{^{32}P \text{ on coverslips} + {}^{32}P \text{ in the uncentrifuged medium}} \times 100$$

Macrophage Associated Viable Bacteria

The experiments were performed as described with the exception that the coverslips were finally put into beakers containing two ml of distilled water Using a rubber eraser the macrophages were rubbed off the coverslips and suspended in the water After 10 min of agitation of the suspension with a Vortex mixer the macrophages had disintegrated From the suspension 1 0 ml was removed for protein determination and 0 5 ml for the counting of viable bacteria The number of viable bacteria was considered equal to the number of CFU after plating of serial dilutions on solid media

Visual Counting of Bacteria in the Macrophages

Experiments were performed as described above At pre set intervals the coverslips were fixed in ethanol and stained with Giemsa stain On each coverslip the number of bacteria per macrophage was counted in at least 200 randomly chosen macrophages

Growth of E coli *and Release of ³²P in the Absence of Macrophages*

E coli labelled with ^{32}P was grown in Leighton tubes at 37 °C and 5 per cent CO_2 The medium was supplemented with 30 μg per ml of bovine serum albumin to compensate for the protein content of disrupted and detached macrophages The growth of *E coli* as CFU and ^{32}P activity in the medium before and after centrifugation at 10000xg for 10 min was determined at zero 1 2 3 and 5 hours of incubation The release of ^{32}P from the bacteria into the medium was expressed as the ratio of ^{32}P activity after and before centrifugation

RESULTS

The tables and illustrations are based on results of selected typical experiments Experiments were performed three to five times and mostly with three parallels

Release of Radioactivity from ³²P labelled E coli *to the Medium by MPM*

Soon after ingestion of ^{32}P labelled *E coli* by MPM ^{32}P labelled molecules are degraded and soluble ^{32}P is released to the medium Fig 1 shows the time dependent release of soluble ^{32}P to the medium and the decrease of macrophagebound ^{32}P in the post ingestive period The curves represent the medians of one experiment performed in triplicate

Spontaneous release of ^{32}P from labelled *E coli* cultivated in KRG medium in the absence of macrophages is regarded as a background activity This spontaneous release was 14 per cent after 5 hours of cultivation in contrast to the MPM mediated release which was between 50 and 60 per cent during the same period (Table 1)

In Table 2 the distribution of label in one experiment with 8 parallels is recorded

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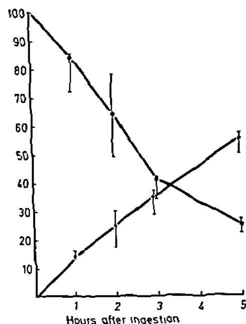


Fig 1 Release of radioactivity from ^{32}P labelled *E coli* by mouse peritoneal macrophages (MPM) MPM on coverslips in Leighton tubes were exposed to ^{32}P labelled *E coli* (10^9 bact per ml) for 30 min and subsequently incubated for 20 min in fresh medium to complete internalization of bacteria Coverslips were then transferred to new media and release of radioactivity to the medium macrophage bound radioactivity and protein content of the macrophages were assayed after 0 1 2 3 and 5 h of incubation The figure illustrates the release of ^{32}P to the medium (▲—▲) and decrease of macrophage bound ^{32}P (●—●) Vertical bars indicate the range Total radioactivity in each Leighton tube is set to 100 per cent The curves indicate the median of one experiment performed in triplicate

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Growth of ^{32}P labelled *E coli* ($3\,5 \times 10^6$ per ml) after incubation for 4 periods of time at 37 °C in 5% CO_2 Release of radioactivity to the medium determined as the quotient (%) of radioactivity after and before centrifugation of the suspension at 10000x*g*

lar multiplication on one side and the bactericidal activity of MPM on the other The results from an experiment in which the number of viable bacteria was checked during incubation is shown in Table 3 During the post ingestive period of 5 hours the number of viable bacteria was reduced to one third of the initial number

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Microscopical examination of Giemsa stained preparations after various times of incubation revealed that the mean number of bacteria associated with macrophages was practically constant during the experimental period (Table 4)

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TABLE 2 *Release of Radioactivity from Phagocytized ^{32}P labelled* E coli *Accuracy of the Experimental System*

Time (h)	Macrophage bound ^{32}P activity (CPMM × 10^3)[a]	Release of ^{32}P to the medium[b]	Protein content of the coverslips (µg)
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3	48 (40– 54)	38 9 (34 8–44 8)	27 0 (24 0–33 6)

[a] Counts per mg cell protein and min
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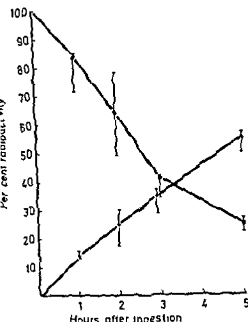


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Release of Radioactivity from ^{32}P-labelled E. coli *to the Medium by MPM*

Soon after ingestion of ^{32}P-labelled *E. coli* by MPM, ^{32}P labelled molecules are degraded and soluble ^{32}P is released to the medium. Fig. 1 shows the time-dependent release of soluble ^{32}P to the medium and the decrease of macrophagebound ^{32}P in the post-ingestive period. The curves represent the medians of one experiment performed in triplicate.

Spontaneous release of ^{32}P from labelled *E. coli* cultivated in KRG medium in the absence of macrophages is regarded as a background activity. This spontaneous release was 14 per cent after 5 hours of cultivation, in contrast to the MPM mediated release which was between 50 and 60 per cent during the same period (Table 1).

In Table 2 the distribution of label in one experiment with 8 parallels is recorded.

Survival of E. coli *Associated with MPM*

The number of MPM-associated, viable bacteria during incubation will be the resultant of intracellu

noted by others *Iwata et al* *Beckerdite et al* and *Friedlander* have reported that some Gram positive and Gram negative bacteria became nonviable before destruction of their cell wall was evident (6 1 5)

The intracellular killing of bacteria is accompanied by a rapid increase in the permeability of the bacterial envelope which renders the molecules of the bacterial cytoplasm open to attack by the digestive enzymes of the phagocytes (1 4)

Degradation of microbial molecules and subsequent release of molecular markers to the culture medium have been used to quantify killing including measurement of released radioactivity from microorganisms prelabelled with various radioactive isotopes

The time-dependent release of isotopes depends on the classes of molecules they label The extensive studies of *Cohn* (2) on the fate of phagocytized ^{32}P labelled bacteria showed that the three major classes of molecules labelled by this marker are proteins RNA and DNA He also documented that the proteins are degraded first followed by RNA and DNA and that the release of ^{3} P starts immediately after the ingestion of the bacteria The minimal lag between killing event and release of label is confirmed in our study (Fig 1 Table 3)

Friedlander (5) observed that the release of radioactivity from bacterial DNA labelled with ^{14}C thymidin could be directly correlated to bacterial death There was however a substantial lag between the killing and the release of label The use of a label that is released shortly after the killing event makes the system more sensitive but also less specific (5) When factors influencing the killing are studied a sensitive system is mandatory

Re incorporation of released label re ingestion of bacteria liberated by egestion or disruption of macrophages represent possible sources of *error* when radio-labelled bacteria are used to measure phagocytosis or intracellular killing We have previously reported that this is of minor importance

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...n grown in the absence of macrophages the spontaneous release of radioactivity from ^{32}P labelled *E col* was 14 per cent after 5 hours of cultivation The release of radioactivity *from* macrophage associated bacteria was 50–60 per cent during the same period (Fig 1 *Table 1*)

Fig 2 shows that in the post ingestive *period* the medium contains soluble ^{3} P and ^{32}P that can be pelleted at 10 000xg for 10 minutes *Friedlander* (5) documented in his experiments that *almost all* soluble radioactivity remained in the supernatant when the culture medium was centrifuged as described These results are in agreement with the findings of *Cohn* (2) on the fate of bacteria ingested by phagocytic cells He demonstrated that ^{32}P labelled molecules of phagocytized bacteria were degraded and excreted to the medium as small soluble molecules The amount of soluble ^{3} P in the medium corresponded to the bacterial breakdown in the phagocyte (2)

Rowley (12) could not demonstrate that MPM had any cidal effect on non-opsonized *E coli* *Steigbigel et al* (14) found that human monocytes were able to phagocytize and kill non-opsonized *E coli* *Staph aureus* and *Salmonella* sp *Men et al* (7) observed that human monocytes phagocytized and killed non-opsonized *E coli* *Midtvedt & Baardsen* (8) using the same strain of *E coli* as used in our work found that rat polymorphonuclear leukocytes killed opsonized as well as non-opsonized bacteria at approximately the same rate as our MPM

It has been argued that polymorphonuclear and mononuclear phagocytes have the same *killing* capacity and that the intracellular killing *depends* more on the microbial species than on the type and source of phagocytes (10 14) *Preliminary data in* our laboratory indicate that the time lag between attachment and complete internalisation of non opsonized *E coli by* MPM *is about 15* minutes (to be published later) To ensure that all bacteria were

investigators may be due to a slower rate of internalisation (10 14)

Mononuclear phagocytes have at least three functional receptors for the recognition attachment *and subsequent* internalization of microorganisms the Fc *receptor* *the complement* receptor and the non-specific receptor (13) Our *in vitro* studies strongly indicate that non-opsonized *E coli* is internalized via the non specific receptor and *subsequently* killed and destroyed by MPM An opson...

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de... ...ur method provides results of consistent accuracy and therefore should be well suited for the *study* of factors influencing the killing capacity of MPM

TABLE 3 *Viability of Macrophage Associated* E. coli *in the Post Ingestive Period*

Time (h)	Bacteria per mg cell protein × 10^8
0	2 2 (2 1–2 3)
1	1 4 (1 1–1 5)
2	0 9 (0 7–1 2)
3	1 1 (1 1–1 0)
5	0 8 (0 8–0 9)

Macrophages on coverclips were exposed to *E coli* (10^9 bacteria per ml) for 30 min and subsequently incubated in fresh medium for 20 min to complete internalization of bacteria Macrophages were disintegrated by hypotonic lysis after 0 1 2 3 and 5 h of further incubation Bacteria were counted as CFU and the protein content of the macrophages assayed The results are expressed as CFU per mg cell protein Median and range from one experiment performed in triplicate

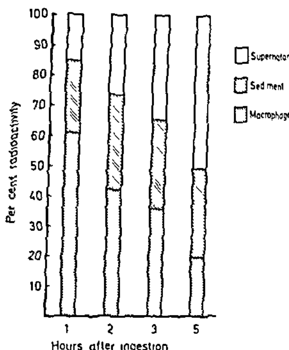


Fig 2 Distribution of radioactivity (per cent of total activity) among MPM suspending medium and sediment when ^{32}P labelled *E coli* had been phagocytized by the MPM and the suspending medium centrifuged at $10000 \times g$ for 10 min Results from one experiment presented in Fig 1

lips Radioactivity in the medium thus represents not only soluble ^{32}P from disintegrated bacteria but to some extent also ^{32}P bound to detached MPM and bacteria The distribution of radioactivity between macrophages on coverslips soluble ^{32}P in the medium and particle bound radioactivity in the medium is visualized in Fig 2 The radioactivity associated with the macrophages gradually decreased as the radioactivity in solution increased Radioactivity associated with detached macrophages was increasing only slightly

DISCUSSION

The majority of bacteria are killed rapidly after ingestion by phagocyres (2 5) The most common way to determine the killing of bacteria by phagocytes has been to determine the time-dependent decrease in the viability of phagocyteassociated bacteria as a decrease in CFU Owing to aggregation of bacteria during the necessary lysis of the phagocytes counting of CFU is subject to considerable sampling errors (5) Multiplication of bacteria within the phagocytes or in the medium after release from disrupted macrophages is also a possible source of error in such assays

We tried to overcome some of the problems by counting only the bacteria bound to macrophages left on the coverslips and then calculating bacteria per mg cell protein (Table 3) When assayed this way intracellular killing of bacteria was almost 50 per cent within one hour of complete ingestion

The decrease in macrophage associated viable bacteria must be correlated to the mean number of bacteria per macrophage counted visually In our experiments the mean number of bacteria was almost constant during the post ingestive period (Table 4) This indicates that the decrease in CFU per mg cell protein observed is due to the bacteria being killed or suffering reduced viability A possible intracellular multiplication can not be ruled out

The absence of morphological alterations of bacteria in the early post ingestive period is also

TABLE 4 *Visual Counting of Bacteria per Macrophage*

Time (h)	Mean number of bacteria per macrophage			
0	19 8	6 7	5 4	9 5
1	23 0	6 8	4 9	9 9
2	23 4	6 5	4 9	9 1
3	20 7	6 3	4 7	9 7
5	22 4	6 2	4 ?	9 4

Macrophages were exposed to *E coli* (10^9 bact per ml) for 30 min and subsequently incubated in fresh medium for 20 min to complete internalization of bacteria Coverslips were fixed in ethanol and stained after 0 1 2 3 and 5 h of further incubation The number of bacteria in about 200 macrophages were counted on each coverslip Results from four experiments each with one or two parallels

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CHARACTERIZATION OF THE BACTERICIDAL ANTIBODY RESPONSE AGAINST *HAEMOPHILUS INFLUENZAE*

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Institute of Medical Microbiology University of Goteborg Goteborg Sweden

Dahlberg T & Branefors P Characterization of the bactericidal antibody response against *Haemophilus influenzae* Acta path microbiol scand Sect. C 88 115–120 1980

Rabbits were used in a study of the bactericidal (BC) antibody response against capsulated *Haemophilus influenzae* of types a and b and their respective non-capsulated variant strains Previously described methods complement fixation and indirect hemagglutination were used for comparison A bactericidal antibody response was demonstrable within a week after the primary immunization against capsulated as well as non capsulated bacteria After the hyperimmunization course the BC titres against non capsulated bacteria were about 1 4 000 while the BC titres against the capsulated – types a and b– strains reached levels of no less than 1 130 000 Samples of separated hyperimmune sera showed BC activity in IgM as well as IgG-containing fractions whereas the BC activity of primary response samples was demonstrable mainly in IgM-containing fractions The BC effect on non-capsulated bacteria of primary response samples were more heat labile than those obtained after hyperimmunization In immune sera a crossreactive BC activity was demonstrable on non-capsulated bacteria the titre being 32 64 fold lower than that of the homologous activity

Key words *Haemophilus influenzae* capsulated non-capsulated bactericidal antibodies

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It has been known since 1932 from the work of *Ward & Wright* that a BC effect could be produced on capsulated *H influenzae* by immune serum containing complement (15) The BC effect of anticapsular antibodies is well documented as reported in an extensive review by *Solotorovsky & Lynn* in 1978 (14) In addition antibodies against somatic antigens can probably be bactericidal since absorption with purified capsular substance did not eliminate all BC effect (1 9 10 13) Whether both kinds of antibodies may contribute to protection is still an open question (14)

In a previous study we have established suitable experimental conditions in rabbit sera for testing complement-dependent BC activity against capsulated as well as non-capsulated *H influenzae* (5) The results showed that a heat stable (56 °C 5 min) BC effect on capsulated *H influenzae* was obtained only in sera from immunized animals while the non capsulated bacteria were also sensitive to preimmune sera the effect being eliminated by heating to 56 °C for 5 min

The aim of the present investigation was to study the development of BC antibodies of IgM as well as IgG class against capsulated and non-capsulated *H influenzae* in rabbits after primary and booster injections The use of capsulated bacteria and non capsulated variants made it possible to study the BC effect of antibodies directed against the capsular and the cell wall antigens respectively

MATERIALS AND METHODS

Strains

H influenzae type a strain Smith (Ma) *H influenzae* type b strain RAB (Mb) and their respective non capsulated variants (Sa and Sb) were used in this study as well as in the previous studies (2 3 5)

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MATERIALS AND METHODS

Strains

H influenzae type a strain Smith (Ma) *H influenzae* type b strain RAB (Mb) and their respective non capsulated variants (Sa and Sb) were used in this study as well as in the previous studies (2 3 5)

Antisera

Immunization procedure Serum samples from the rabbits used in a previous study were employed *in this* investigation too For each of the four strains two rabbits were given a primary immunization with 10^8 bacteria and four weeks later a booster dose of the same amount After another three weeks a hyperimmunization course was started and each rabbit was given a total of 2–4 × 10^{10} bacteria The immunization schedule *and the* bleedings performed are indicated in Figs 1 and 3

A second series of rabbits were immunized with Sa and Sb bacteria two rabbits for each *strain A primary* dose of about 10^8 bacteria was given and eight weeks later a booster dose of about the same amount After another six weeks 2–3 further injections *were given* within a period of 4 weeks Each of these rabbits was given in total 4–5 × 10^8 bacteria

Heat treatment of sera was performed by incubating serum samples in a waterbath at 56 °C for 5 min and 30 min

Separation of sera was performed by filtration of 4 ml serum on a 77 × 2 5 cm column of agarose (Biogel A5 m Bio Rad Lab Richmond Calif USA) phosphate (0 05 M) buffered saline (0 5 M) pH 7 being used for elution The flow rate was 10 ml/h The optical density (OD) of each 5 ml fraction was measured at 280 nm on a Beckman DB spectrophotometer The fraction were tested for IgM and IgG content by double diffusion in agar employing antisera specific to the Fc part of rabbit IgG and IgM heavy chain (Nordic Immunological Laboratories Tilburg The Netherlands)

Serological Methods

Bactericidal assay (BC) was performed on twofold serial dilutions of sera or on serum fractions with guinea pig serum (dilution 1 20 for Sa and Sb undiluted or dilution 1 2 for Ma and Mb) as source of complement (C) as previously described (5)

Indirect hemagglutination assay (IHA) was used to measure antibodies against the capsular antigen and *complement fixation* (CF) with bacterial suspension of Sa and Sb bacteria as antigen was used to measure antibodies against O antigens and other cell wall antigens as described previously (4)

All serum samples obtained from each rabbit were tested on the same occasion for each assay

RESULTS

Antibody Response During Immunization with Ma and Mb Bacteria

The results summarized in Fig 1 show that a capsular antibody response was demonstrable with IHA four days after the primary immunization reaching maximum levels after one to three weeks The booster dose caused only a slight and short titre increase whereas the hyperimmunization gave rise to a pronounced incresae in IHA titres

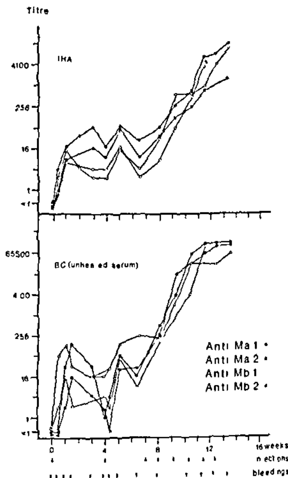
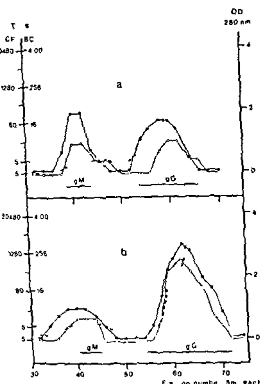


Fig 1 Development of hemagglutinating (IHA) and bactericidal (BC) antibodies during the immunization course for the two anti Ma and the two anti Mb rabbit sera

No BC activity was observed before immunization but four days to one week after the primary immunization a BC effect was demonstrable The hyperimmunization course resulted in titres rapidly increasing to very high levels the final titres being about 1 131 000 (Fig 1) The BC activity of the heated primary response antiserum samples remained unchanged after heating to 56 °C for 30 min

In A-5 m separated serum samples obtained one week after the primary immunization low BC titres in IgM containing fractions were demonstrable for both the anti Ma and anti Mb sera while IHA titres were not demonstrable (Fig 2 a) Samples of ... against Ma and Mb ... ctions as ... ng much higher BC titres than the former In IgM containing fractions the IHA titres far exceeded the BC titres while the reverse was true for the IgG-containing fractions (Fig 2 b)

The antibody response of the anti Ma and anti Mb sera against corresponding non-capsulated



F g 2 IHA and BC titres of anti Mb serum 1 samples obta ned in IgM and IgG fractions of A5 m agarose separated serum
a) serum sample one week after pr mary immunization
b) f nal serum sample
Gu nea p g serum n d lut on 1 2 added as source of C
The OD at 280 nm of the fractions is marked by the dotted l ne

TABLE 1 *Final Bacter cidal Titres of the E ght Rabb t Ant sera Tested with Ma–Sa and Mb–Sb Bacter a Respect vely*

Test bacter a		anti Sa	ant Ma
Sa	1	1 2048	1 2048
	2	1 4100	1 2048
Ma	1	1 128	1 131000
	2	1 256	1 131000
		anti Sb	anti Mb
Sb	1	1 8200	1 2048
	2	1 16400	1 4100
Mb	1	1 2048	1 131000
	2	1 4100	1 131000

variant showed that CF titres and BC activity (stable to 56 °C 5 min) were demonstrable after the primary immunization The CF and BC titres during hyperimmunization were comparable to those of the anti Sa and anti Sb sera respectively The final BC titres are given in Table 1

Antibody Response During Immunization with Sa and Sb Bacteria

The results summarized in Fig 3 show that in the preimmune sera CF antibodies against Sa and Sb bacteria were not demonstrable while 2–4 days after the primary immunization CF antibodies were revealed with maximal titres after about one week The CF titres after the booster dose were of the same magnitude as those after the primary immunization During the hyperimmunization course the titres gradually increased about 16 fold for the anti Sa and about 64 fold for the anti Sb sera

There was BC activity against Sa and Sb bacteria in the unheated preimmune sera and the primary and booster immunizations did not cause any significant titre increase (≧fourfold) During the hyperimmunization course a a gradual titre increase was obtained resulting in 64 to 256 fold higher titres in the final serum samples than in the preimmune sera (Fig 3 and Table 1)

Whereas the preimmune sera had no BC effect on Sa and Sb bacteria after heating to 56 °C for 5 min the anti Sa as well as the anti Sb serum samples obtained 2–4 days after the primary immunization retained their BC effect after heating to 56 °C for 5 min (Fig 3) When heating was extended to 30 min however no BC activity could be detected until 1–2 weeks after the hyperimmunization course had started The titres than rose within two weeks to about the same levels as those of unheated serum samples (Fig 3)

In A5 m separated samples of preimmune sera neither CF titres nor BC activity was demomstrable against Sa or Sb bacteria However in the A5 m separated samples from the anti Sa sera obtained one week after the primary immunization CF titres and BC activity was demonstrable in both IgM and IgG-containing fractions (Fig 4 a) The correspon ding anti Sb sera showed CF titres and BC activity only in IgM-containing fractions

In the f nal samples of bo h h ... r

IgM and IgG-containing fractions from primary response samples retained their BC activity after heating to 56 °C for 30 min in contrast to the whole serum samples However when such a serum sample was first heated to 56 °C for 30 min and

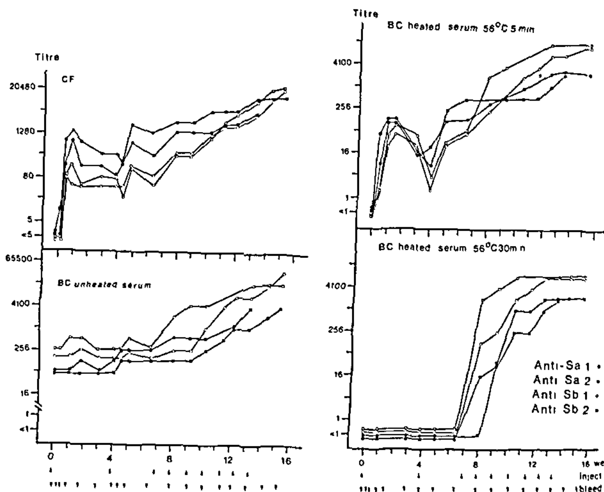


Fig 3 Development of complement fixing (CF) and bactericidal (BC) antibodies during the immunization course for the two anti Sa and the two anti Sb rabbit sera

then separated, no BC activity was demonstrable in *any fraction*

A BC effect of the anti-Sa and anti-Sb sera against Ma and Mb bacteria respectively was *demonstrable only in samples obtained from the* latter part of the hyperimmunization period The BC titres of the final samples of the anti Sa sera against the heavily capsulated Ma bacteria were considerably (16-fold) lower than against homologous Sa bacteria, while the BC titres of anti Sb sera against Mb bacteria, which has ordinary thick capsule were only fourfold lower than against homologous Sb bacteria (Table 1)

Crossreactive Bactericidal Activity

Samples from both anti Sa and anti-Sb sera were tested against bacteria of the heterologous capsulated and non-capsulated strains in order to reveal a possible BC effect It was found that heated serum samples (56 °C, 5 min) obtained from hyperimmunized *animals* (given about 2–4 × 10^{10} bacteria) as well as from the second series of animals, given only about 4 × 10^8 bacteria, had a BC effect on *heterologous non-capsulated bacteria The homologous* BC titres of the anti-Sa and the anti Sb sera were 32-64-fold higher than the heterologous titres which *ranged between 1 16 and 1 128 Fig* illustrates a representative experiment involving anti-Sa serum and Sa and Sb bacteria The figure also illustrates the gradual decrease in BC effect generally observed on heterologous bacteria with *increasing serum dilutions*, as opposed to the rapid decline of the homologous BC effect

DISCUSSION

The results of studies on antibody response against capsulated *H influenzae* revealed antibodies against capsular polysaccharide within a week *after the* primary immunization by means of IHA as well as ELISA (to be published) The induced antibodies *showed BC activity and were of IgM class* indicating a primary type of response The serum

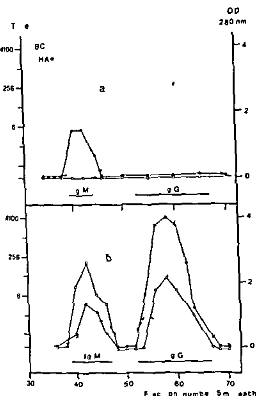


Fig 4 CF and BC titres of anti Sa serum 1 samples obtained in IgM and IgG fractions of A5 m agarose separated serum
a) serum sample one week after primary immunization (unheated)
b) final serum sample
Guinea pig serum in dilution 1 20 added as source of C
The OD at 280 nm of the fractions is marked by the dotted line

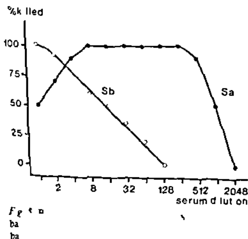


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samples obtained after hyperimmunization showed very high BC titres mainly of IgG class The IHA titres were somewhat higher in IgM than in IgG containing fractions an indication that this method is much more sensitive for measuring IgM class antibodies than those of IgG class A distribution of IHA and BC activity in the IgM and IgG fractions similar to that in the rabbit hyperimmune sera has been reported for a human serum after a single immunization with *H influen ae* type b capsular substance (7) The secondary type of response after a single injection of antigen may be explained by the existence of an immunological memory for *H influen ae* polysaccharides in human adult individ uals

The studies on antibody response against non capsulated *H influen ae* revealed antibodies within a week after the primary immunization using bacterial suspensions as antigen for a CF test as well as purified lipopolysaccharide from non-capsulated *H influen ae* (LPS) as antigen for ELISA (to be published) A BC effect of these antibodies was possible to observe by heating serum samples to 56 °C for 5 min this treatment destroyed the BC effect on non-capsulated *H influenzae* observed in preimmune rabbit sera (5) The presence of specific antibodies in primary response samples was further supported by the finding that in A5 m separated sera IgM and for anti Sa also IgG-containing fractions CF and ELISA LPS titres (unpublished) were demonstrable together with a BC effect

The BC activity of primary response antibodies against non-capsulated bacteria was found to be abolished by heating whole serum to 56 °C for 30 min while in A5 m separated serum samples the BC activity of IgM and IgG antibody containing fractions were not affected by this treatment In contrast the hyperimmune sera against non capsulated bacter a re a ned h BC

u c experiments performed no explanation can be given for the discrepancy between non-capsulated and capsulated bacteria concerning the heat lability of the primary response BC effect

As a practical consequence of these observations for measuring BC antibodies it might be advisable to heat sera to 56 °C for no longer than 5 min since this treatment is sufficient for destroying the BC effect on *H influenzae* observed in preimmune sera (5) probably caused by the alternative pathway of C activation

Concerning the specificity of the antibod es those directed against the capsular antigen appear to be

bactericidal The results of the present investigation support those of previous studies (14) The BC method appears to be very sensitive for measuring anti capsular antibodies, a BC effect of the primary response being demonstrable in IgM-containing fractions in which IHA titres could not be revealed

Samples of antisera against the non capsulated Sa and Sb bacteria taken during the primary immunization were not bactericidal for the corresponding capsulated bacteria (Ma and Mb respectively) while samples taken from the latter part of the hyperimmunization course were bactericidal for capsulated bacteria of the respective strain This might indicate that antibodies directed against cell wall antigens can penetrate the capsulae causing a BC effect provided their avidity is high enough This suggest that antibodies against cell wall antigen(s) may contribute to protection against capsulated bacteria as well (14)

In order to reveal which cell wall antigen(s) are the target for antibodies with a BC effect further investigations are needed using, e g absorbed antisera and antisera against various cell wall preparations The two strains used Smith and RAB have by previous immunodiffusion analyses been shown to have different LPS side chains (O antigens) (3) and antibodies against the O antigen are probably bactericidal as proposed to enterobacteria (8 11 12) The findings that a heterologous BC effect of antisera against non capsulated bacteria was observed might be an indication that a common part of LPS (6) and/or other cell wall antigens are involved as well

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INFLUENCE OF AN INTESTINAL MICROFLORA ON THE DEVELOPMENT OF THE IMMUNOGLOBULINS IgG1, IgG2a, IgM and IgA IN GERM-FREE BALB/c MICE

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Department of Physiology University of Odense and Gammel Bomholtgård Laboratory Animals Breeding and Research Center Ry Denmark

Nielsen E & Friis C W Influence of an intestinal microflora on the development of the immunoglobulins IgG1 IgG2a IgM and IgA in germ free BALB/C mice Acta path microbiol scand Sect C 88 121–126 1980

The development of immunoglobulins IgG1 IgG2a IgM and IgA was studied in germ free BALB/c mice during the first 14 weeks of life A transfer of maternal IgG1 and IgG2a was demonstrated during the first 2 weeks of this period Endogenous synthesis of immunoglobulins was found only for IgM the level of which was significantly higher at the end of the period than in a conventional group After oral association of germ free BALB/c mice with an intestinal hexaflora the serum concentrations of IgG1 IgG2a and IgA increased The levels of IgA and of IgG1 were higher in the associated group than in the conventional group

Key words Immunoglobulins germ free BALB/c mice intestinal microflora

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Studies of the immunoglobulins of germ free mice have revealed lower serum concentrations of IgG and IgA in these animals than in their conventional counterparts (*Fahey & Sell* 1965 *Crappe et al* 1969 *Kiuchi et al* 1972) while higher concentrations have been demonstrated for the IgM class (*Arnason et al* 1964 *Crabbe et al* 1969)

The ontogeny of the immunoglobulins in germ free mice has been studied by *Kiuchi et al* (1972) who demonstrated low concentrations of IgG during the first two weeks of life while these immunoglobulins could not be demonstrated from the 3rd to the 25th week The IgM and IgA classes were not studied

The demonstrated differences in the immunoglobulins between germ free and conventional mice are generally attributed to the absence of an intestinal microflora in the germ free mice So far a systematic study of the development of the immunoglobulins IgM IgA and sub-classes of IgG in germ free mice has not been undertaken neither has the influence of an oral stimulation with an intestinal microflora on this development been studied

This study was conducted to elucidate the development of the [illegible]
IgM and [illegible]
influence [illegible]
microflora [illegible]

MATERIALS AND METHODS

Mice

All mice were of the BALB/c/ABOMf strain The germ free mice were kept in isolators at Gammel

bactericidal The results of the present investigation support those of previous studies (14) The BC method appears to be very sensitive for measuring anti-capsular antibodies, a BC effect of the primary response being demonstrable in IgM-containing fractions in which IHA titres could not be revealed

Samples of antisera against the non-capsulated Sa and Sb bacteria taken during the primary immunization were not bactericidal for the corresponding capsulated bacteria (Ma and Mb, respectively) while samples taken from the latter part of the hyperimmunization course were bactericidal for capsulated bacteria of the respective strain This might indicate that antibodies directed against cell wall antigens can penetrate the capsulae causing a BC effect provided their avidity is high enough This suggest that antibodies against cell wall antigen(s) may contribute to protection against capsulated bacteria as well (14)

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RESULTS

IgG1

The mean values for IgG1 and the standard error of the means observed at birth and during the first 14 weeks of life in germ free associated and conventional BALB/c mice are shown in Fig 1

The germ free group showed a level of about 10 au/l at birth Moderate levels of IgG1 were detected during the first 16 days and thereafter they declined during the following two weeks

The associated and the conventional groups showed similar levels at birth succeeded by an increase and a decline similar to those in the germ free group At the end of the period the levels of IgG1 for both the associated and the conventional groups were at 50 and 30 au/l respectively On day 49 84 and 98 the mean IgG1 levels of the associated group were significantly higher (Student s t test $p < 0.02$ Wilcoxon s test $0.01 < p < 0.05$) than the level of the conventional group

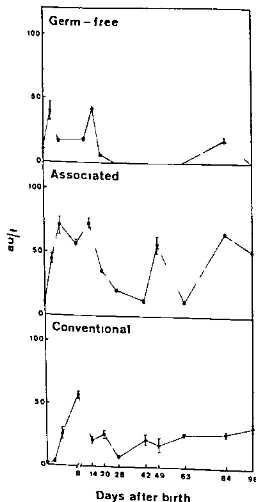


Fig 1 Levels of IgG1 in germ free associated and conventional BALB/c mice during the first 14 weeks of life Each point represents the mean and the standard error of the mean for the group

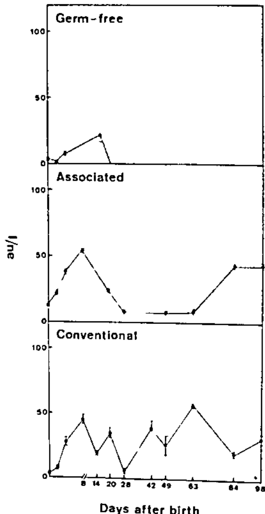


Fig 2 Levels of IgG2a in germ free associated and conventional BALB/c mice during the first 14 weeks of life Each point represents the mean and the standard error of the mean for the group

IgG2a

The levels of IgG2a are shown in Fig 2 In the germ free group a level of about 4 au/l was found at birth During the first 16 days the levels ranged between 0 and 16 au/l After the 16th day no IgG2a was demonstrated

In the conventional and associated groups the

Bomholtgård Laboratory Animals Breeding and Research Center under controlled germ free conditions Routine examinations of the mice were negative for bacteria fungi and parasites Conventional controls randomly bred during several generations were kept at the Animal Department Odense University

Stimulation Procedure

The oral stimulation was performed as an association with an intestinal hexaflora consisting of *Streptococcus faecalis Lactobacillus brevis Aerobacter aerogenes Staphylococcus epidermidis Bacteroides spurius* and a *Yeast fungus* (Zentralinstitut fur Versuchstierzucht 3 Hannover Linden Germany) Ten pellets of faeces containing the hexaflora were dissolved in 10 ml of sterile water and incubated overnight at 37 °C On the following day ten female mice were given 0 85 ml of the faeces solution in the drinking water The mice were mated with males associated with the same flora and the young which were associated via the faeces from the parents were used in the experiments

Blood Sampling

Blood samples were taken from germ free associated and conventional mice during the first fourteen weeks of life (Table 1)

Antisera

Monospecific antisera against mouse IgG1 IgG2a IgM and IgA were produced by immunization of rabbits with purified myeloma protein The proteins were purified from the ascites fluid of BALB/c mice bearing transplantable plasma cell tumors

Myeloma protein IgM was purified from the ascites of mice bearing a tumor MOPC 104E (*Mc Intire et al* 1965) Ten ml of ascites fluid was fractionated by preparative zone electrophoresis on starch gel gelfiltration chromatography on Sephadex G 200 and ion exchange chromatography on DEAE-cellulose (DE 52 Whatman) The purification procedure was monitored by immuno electrophoresis using rabbit anti total mouse protein antiserum (111SX Dakopatts A/S Copenhagen Denmark) and monospecific antiserum against mouse IgM (B 107 Meloy Laboratories Springfield U S A) The preparation used for immunization showed a single precipitate when tested in crossed immuno electrophoresis against these two antisera

IgG1 and IgG2a were purified from the ascites fluid of mice bearing the tumors MOPC 21A (*Potter & Lieberman* 1967) and LPC 1 (*Potter et al* 1966) respectively IgG containing fractions were obtained by differentiated $(NH_4)_2SO_4$ precipitation as described by *Kalpaktsoglou et al* (1973) and the third precipitate was further fractionated by ion exchange chromatography The fractions selected for immunization showed a single precipitate in crossed immuno electrophoresis using antiserum against total mouse proteins (Dakopatts 111SX) Identity with IgG1 and IgG2a was confirmed by crossed immunoelectrophoresis using antisera against mouse IgG1 (Meloy B 102) and IgG2a (Meloy B 104) respectively

IgA was purified from the ascites of mice bearing a tumor MOPC 315 (*Eisen et al* 1968) by affinity chromatography Anti mouse IgA (Meloy B 106) was coupled to CNBr activated Sepharose 4B Ascites fluid

profile revealed a single peak and crossed immuno electrophoresis using antisera ataınst total mouse proteins (Dakopatts 111SX) and against mouse IgA (Meloy B 106) revealed that the eluted peak was IgA

At intervals of 14 days 75 µl (= 150 µg) myeloma protein + 75 µl Freund s incomplete adjuvant (Difco Laboratories Inc Detroit Mich U S A) were injected subcutaneously into rabbits The rabbit antisera were tested in crossed immuno electrophoresis using antisera with known specifities (Meloy B 102 B 104 B 106 B 107) in an intermediate gel as specifity control

Quantitation of Immunoglobulins

The immunoglobulins were quantified by rocket immuno electrophoresis (*Laurell* 1966a & 1966b) using 1% agarose (Litex HSA Glostrup Denmark) and barbital buffer pH 8 6 ionic strength 0 02 The monospecific antisera were used in concentrations from 5 to 7% The electrophoresis was performed at 2V/cm for 18 hours The wells received 3 µl A reference serum pool defined as containing 100 au/l (arbitrary units) of IgG1 IgG2a IgM and IgA was obtained from 25 adult conventional BALB/c mice The rockets were measured with an accuracy of 0 5 mm giving an uncertainty of 5 to 1% for the used height of 10 to 50 mm

Statistical Methods

The Student s t test and Wilcoxon s rank sum test for unpaired samples were used

TABLE 1 *Numbers of Germ free Associated and Conventional BALB/c Mice Used for Blood Sampling during the First 14 Weeks after Birth*

Days after birth	Numbers of BALB/c mice		
	germ free	associated germ free	conventional
0	3	4	5
2	3	5	6
4	5	5	6
8		4	6
12	3		
14		4	6
16	(		
20	5	4	6
28	6	6	6
42		6	6
49	6	6	6
63	6	6	6
84	3	6	5
98	2	6	6

RESULTS

IgG1

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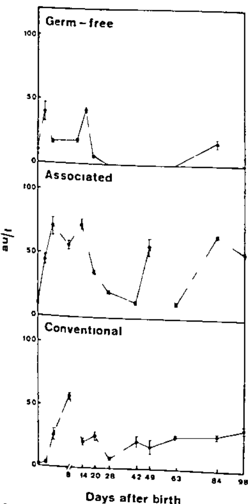


Fig 1 Levels of IgG1 in germ free associated and conventional BALB/c mice during the first 14 weeks of life Each point represents the mean and the standard error of the mean for the group

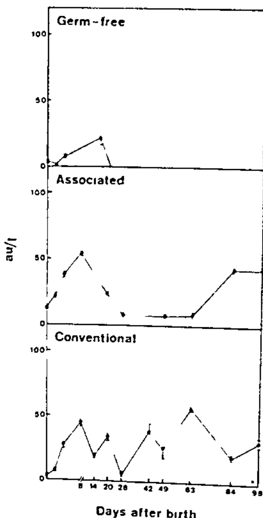


Fig 2 Levels of IgG2a in germ free associated and conventional BALB/c mice during the first 14 weeks of life Each point represents the mean and the standard error of the mean for the group

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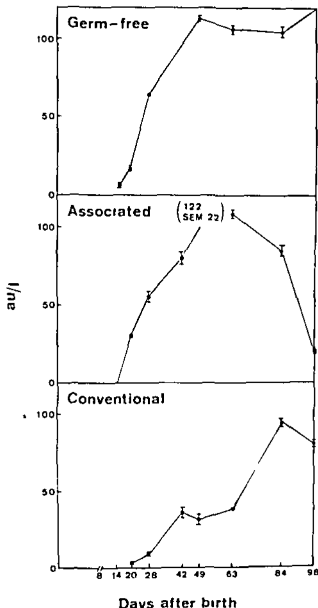


Fig 3 Levels of IgM in germ free associated and conventional BALB/c mice during the first 14 weeks of life Each point represents the mean and the standard error of the mean for the group

levels of IgG2a at birth were between 3 and 13 au/l then increased rapidly during the first 8 to 14 days From the 2nd to the 6th week the level declined in the associated group and then increased during the rest of the period to a level of about 40 au/l For the conventional group the level declined from the 1 t to the 4th week Hereafter an increase during the rest of the period to a level of about 33 au/l was seen

IgM

Weak but visible precipitates indicated the presence of low IgM levels (< 5 au/l) in all groups during the first 14 days (Fig 3) Hereafter the levels increased rapidly The germ free group reached a maximum level of about 130 au/l at the end of the period The associated group showed a maximum of about 120 au/l in the 7th week Then the level declined during the rest of the period to a level of about 20 au/l In the conventional group the level increased to about 80 au/l at the end of the period On day 28 49 and 63 the mean levels of the germ free group were significantly higher (Students t test $p < 0.02$ Wilcoxons test $0.01 < p < 0.05$) than that of the conventional

IgA

In the germ free group only one sample (day 48)

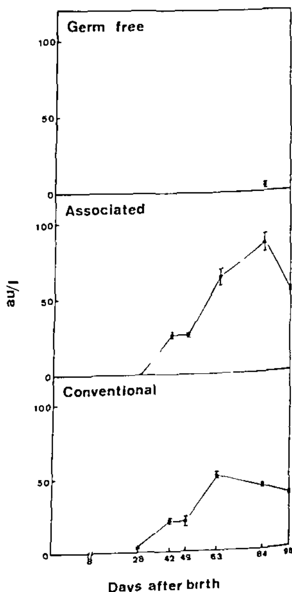


Fig 4 Levels of IgA in germ free associated and conventional BALB/c mice during the first 14 weeks of life Each point represents the mean and the standard error of the mean for the group

shows detectable levels of IgA (Fig 4) In the associated and conventional groups no IgA could be demonstrated during the first 4 weeks After that period the levels showed a moderate increase up to values of about 40–50 au/l During the last 2 weeks the level of the associated group was significantly higher than that of the conventional group (Student s t test $p < 0.02$ Wilcoxon s test $0.01 < p < 0.05$)

DISCUSSION

The development of four immunoglobulin classes was studied in germ free associated and conventional BALB/c mice during the first 14 weeks of life With the exception of the IgM (all groups) and IgA (associated group day 84) none of the mean levels exceeded 70 per cent of the levels of the reference serum The reason is presumably that at the end of the experiment the mice had not yet reached the adults Ig levels

Increasing levels of IgG1 and IgG2a could be demonstrated in the associated and conventional groups during the first 16 days of life In the germ free group the IgG1 level increased more slowly and IgG2a could be demonstrated in very small amounts only Similar observations have been made for conventional mice (*Fahey & Barth* 1965 *Kalpaktsoglou et al* 1973) *Fahey & Barth* (1965) attributed the rapid rise of IgG1 and IgG2a after birth to transfer from the mother via the colostrum and gastrointestinal tract and the declining levels from the 2nd week to a loss of absorptive capacity for the maternal IgG1 and IgG2a This is in agreement with *Lecce* (1972) who demonstrated a selective absorption of γ globulin via the gastro intestinal tract in mice only during the first days of life

From the 4th to the 6th week of life increasing levels of IgG1 IgG2a and IgA are seen in the associated and conventional groups This increase is considered due to an increasing capacity for endogenous immunoglobulin synthesis (*Fahey & Barth* 1965) As no IgG1 IgG2a or IgA could be demonstrated in the germ free group during this period the levels of these classes in the associated group can be attributed to the given microflora Stimulation with the microflora resulted in a level of IgA and possibly IgG1 significantly higher than that of the conventional group at the end of the period

Only negligible amounts of IgM could be demonstrated during the first two weeks Thereafter IgM increased in all groups the germ free mice having significantly higher levels than the conventional ones from the 4th week and during the rest of the period The higher level of IgM in the germ free group is in agreement with the data published by *Arnason et al* (1964)

Both the germ free and the associated groups showed a rapid increase in the level of IgM from the 3rd to the 9th week (Fig 3) Hereafter the level of the associated group declined during the rest of the period while that of the germ free continued to increase This may be due to antigenic stimulation of the germ free group during the whole experimental period and of the associated group during the first 9 to 10 weeks During the first 10 weeks of the experiment both groups were fed with food sterilized by an identical procedure (130 °C in 30 minutes) Hereafter the associated group was given the same food sterilized by heating at 100 °C to 115 °C in 30 minutes *Eggum* (unpublished work) found that a rise in temperature may cause a fall in the protein content of the food Assuming that this loss of protein is a result of a conversion of protein into macromolecular elements with antigenic properties as described by *Sell* (1964) the differences between the germ free and associated animals may be due to a stimulation via the gastro intestinal tract with these elements *Wostmann et al* (1970) attributed the production of antibodies in germ free mice partly to an incomplete elimination of antigenic components from the food and partly to a consumption of excreta from other mice In the present study the declining level of IgM in the associated group during the last 5 weeks of the period indicated that the given microflora had little influence or none on the development of IgM

We wish to thank Dr *M Potter* Laboratory of Cell Biology National Institutes of Health Bethesda Maryland U S A for providing us with the tumors used for this project The skilful technical assistance of Mrs *Inge Andersen* and Mrs *Hanne Holst* is gratefully acknowledged

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SOLID-PHASE ENZYME IMMUNOASSAY OF IgM-CLASS RHEUMATOID FACTOR
COMPARISON OF THREE METHODS FOR PREPARATION OF THE SOLID-PHASE TARGET IgG

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Ziola B & Tuokko H Solid phase enzyme immunoassay of IgM-class rheumatoid factor comparison of three methods for preparation of the solid phase target IgG Acta path microbiol scand Sect C 88 127–130 1980

Three solid phase forms of human IgG were compared for their ability to function as the binding target in an enzyme immunoassay for IgM-class rheumatoid factor (RF) IgG was either directly adsorbed to polystyrene beads (method A) or immunologically (method B) or covalently (method C) bound to protein adsorbed to beads The data presented indicate that C is the method of choice for the preparation of the RF assay solid phase IgG

Key words Rheumatoid factor enzyme immunoassay solid phase IgG configuration

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Several investigators have detected IgM-class rheumatoid factor (RF) with immunoassays which involve binding of RF to a solid phase form of *human* IgG (1 2 4 7) Although different methods were used to prepare the solid phase IgG for these assays no data is available concerning which form of solid phase IgG is best suited as an RF binding target Three methods of preparing the solid phase IgG were compared therefore to determine how the preparation (and thus the final configuration) of the solid phase IgG affects the results of an RF immunoassay

MATERIALS AND METHODS

Isola [illegible]

[illegible] sy[illegible] pr[illegible] 18 % Na_2SO_4 at room temperature (20 °C) The IgG fraction [illegible] then obtained by chromatography on diethylaminoethyl Sepharose CL 6B (Pharmacia Fine Chemicals Uppsala Sweden) equilibrated in 0 1 M tris HCl pH 8 0 at 4 °C Unretained protein was taken to be IgG and the concentration was determined spectrophotometrically by using an extinction coefficient of 13 8 at 280 nm Storage was at −20 °C

Preparation of the Solid phase Human IgG

Specular surface polystyrene beads 6 4 mm in diameter (Precision Plastic Ball Co Chicago U.S.A.) were used as the RF assay solid phase Beads with directly adsorbed immunologically bound and covalently bound IgG were prepared by methods A B and C respectively Addition of 10^{-4} M merthiolate to the IgG solutions permitted each type of bead to be stored for at least three months without discernable effects on the assay results

Method A Beads were placed in 0 02 M *sodium* phosphate pH 7 4 containing 0 14 M NaCl (PBS) and 10 µg of IgG/bead An adsorption for 18 h at 20 °C was followed by storage at 4 °C The beads were removed from the IgG solution as required and either dried in a 20 °C air flow or washed once with PBS When washed the beads were added immediately to the incubation

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TABLE 1 *Effect of Target IgG Configura on on the Sol d Pl ase En me Immun assa of Rheuma oid Factor (RF)*

Sol d phase IgG		OD₄₉ b						Assay
		IU of RF/ml					Pos t v y cut-off	sens t ty threshold
f of on	In dried (D) or hydrated (H) form[a]	131 6	26 3	5 3	1 05	0		(IU of RF/ml)[c]
	D	2 047 (13 8)[b]	1 019 (6 8)	0 459 (3 1)	0 ?20 (1 5)	0 150	0 188	0 5–0 9
adsorption IgG onto d phase	H	1 844 (13 9)	0 943 (6 9)	0 4?? (3 ?)	0 199 (1 5)	0 136	0 170	0 5–0 8
3	D	1 571 (?3 5)[b]	0 714 (10 7)	0 344 (5 1)	0 1?4 (1 9)	0 067	0 084	0 ?–0 3
nolog cal b nd ng IgG to an an n the sol d phase	H	1 756 (?5 8)	0 776 (11 4)	0 395 (5 8)	0 13? (1 9)	0 068	0 085	0 ?–0 3
C	D	1 660 (21 3)[b]	0 778 (10 0)	0 314 (4 0)	0 139 (1 8)	0 078	0 098	0 3–0 4
en binding of gG to a p ote n on the sol d-phase	H	1 930 (25 1)	0 960 (1? 5)	0 395 (5 1)	0 151 (2 0)	0 077	0 096	0 -0 3

he bead we e remo ed f om he r storage solu ons and e her a r-dr ed or r nsed w h PBS just p or to use *in the assays* e age of n ne ntra assay de e m na ons are g ven S nce the intra assay standard devia ons f r all le els of RF w ways less than 8 % the assay pos y cu -off was se a 25 % (a east th ee anda d d

background values These as well as spec f c RF b nd ng as measured by b nd ng ratios did not d ffer if the *solid phase IgG was p e-d ed or lef hydrated* Thus the assay sens t vity thresholds were the *same* n each case be ng approximately 3 fold lower than that obta ned when the RF target IgG was d rectly adsorbed to the sol d phase

Covalent b nd ng of the IgG to a protein base on the sol d phase (method C) came from an attempt to obviate the need to use specif c human sera for prepa n RF

E

F

form ng a covalent bond between the IgG molecules and a prote n base on the sol d phase came *from* a report by *Vestergaard e al* (6) in wh ch glutaralde hyde was used to l nk v ral antigen to bov ne serum album n adsorbed to a plast c sol d phase Periodate activated fetu n was used in the present experi *ments since t was felt that this procedure* is more reproducible n prepar ng a sol d phase wh ch has *large* amounts of RF target IgG However no d rect *comparison* was made with coupl ng of the IgG to a protein base with glutaraldehyde

Preparat on of the sol d phase IgG by method C was found to result in sl ghtly increased assay *background* values compared to preparation by m h D

d the beads were left hydrated In this case the assay sens tivity threshold was *equal* to that obta ned w th the sol d phase IgG prepared by method *B* Although drying of the sol d phase IgG

tubes to prevent dehydration of the surface bound IgG

Method B Beads with human IgG in the form of immune complexes were prepared as described pre viously (7) except that 10 μg of IgG from the present human serum pool were required to sensitize each antigen coated bead Beads were incubated with a membraneous antigen prepared from respiratory syncy tial virus infected cells and then dried Virus specific antibodies from the purified human IgG were allowed to bind to antigen during a 24 h incubation at 20 °C The beads were then stored at 4 °C in the IgG solution and used in the assays either pre dried or still hydrated (see method A)

Method C Beads were placed in PBS containing 10 μg foetal calf fetuin (type III Sigma Chemical Co St Louis USA) per bead After 18 h at 20 °C the solution was aspirated and the beads were dried IgG was then covalently bound to the carbohydrate moiety of the bead adsorbed fetuin (5) The fetuin coated beads were incubated for 1 h at 20 °C in PBS containing 0 05 M $NaIO_4$ The beads were then rinsed three times with 0 1 M sodium carbonate pH 9 5 and covered with the same buffer containing 10 μg of IgG/bead A 24 h incubation at 20 °C was followed by storage at 4 °C When required beads were removed from the IgG solution and placed in carbonate buffer containing 0 05 $NaBH_4$ The reducing solution was aspirated after 1 h at 4 °C and the beads were rinsed twice with PBS The fetuin coated beads with covalently bound IgG then used in the assays either predried or still hydrated (see method A)

RF Assay Procedure

The assays were performed as described previously (7) except that enzyme conjugated rather than ^{125}I labelled RF indicator antibodies were used

Serum specimens were tested in a dilution of 1 200 and RF bound to the solid phase IgG was detected with horse radish peroxidase conjugated porcine IgG specific for the μ polypeptide of human IgM (Orion Diagnostica Helsinki Finland diluted 1 800) Following aspiration of unbound RF indicator antibodies and two washes with tap water the damp beads were rolled into tubes containing 0 5 ml of enzyme substrate solution (0 1 M citrate/phosphate pH 5 5 containing freshly dissolved 3 mg/ml *o* phenylenediamine and 0 02 % H_2O_2) After 30 min at 20 °C the enzyme reaction was stopped with 0 5 ml of 1 N HCl and the OD_{492} of each sample was read in a nine channel spectrophotometer (FP 9 Finnpipette Labsystems Oy Helsinki Finland) The HCl diluted enzyme substrate solution used to blank the machine always had an OD_{492} of < 0 050 when checked against H_2O

The rheumatoid arthritis serum pool employed in this study had previously been assigned an arbitrary value of 1 000 units of RF/ml This serum pool was found subsequently to contain 131 6 IU of RF/ml by comparison with in International Reference Preparation of Rheumatoid Arthritis Serum (International Labora tory for Biological Standards Statens Seruminstitut Copenhagen Denmark) Samples containing known levels of RF were prepared by making dilutions of the serum pool with the diluent used in the assays

RESULTS AND DISCUSSION

The three methods used to prepare the solid phase IgG result in the IgG having different configura tions Direct adsorption of the IgG to the beads (method A) probably causes the Fc portion of most of the molecules to be contiguous with the solid phase This kind of RF target IgG was used in the RF immunoassays described by *Carson et al* (1) and by *Knez & Reimer* (2) When the RF target IgG is immunologically complexed to an antigen base (method B reference 7) the IgG probably is elevated from the solid phase with the Fc regions of most of the molecules extended towards the aqueous phase Preparation of the solid phase IgG by method C results in an intermediate situation The RF target IgG is again elevated from the solid phase but the covalent bound to the protein base need not be with the Fab region of the IgG Therefore the IgG may have either the Fab or the Fc regions extended towards the aqueous phase The solid phase IgG employed in the RF enzyme immunoassay of *Maiolini et al* (4) probably has such a configuration even though the IgG probably is closer to the solid phase due to direct binding of the IgG to surface reactions groups Since drying might alter the configuration of the RF target IgG each of the three solid phase IgG forms was evaluated in a pre dried and a hydrated state

phase IgGs within a

each case significant differences exist in the results obtained One important difference is that direct adsorption of the RF target IgG to the beads resulted in elevated enzyme-control values This was found consistently and may stem from rearrangement (i e denaturation) of the IgG secon dary or tertiary structure upon adsorption to the plastic solid phase (3) Due to the elevated background values the assay sensitivity threshold was higher than when methods B or C were used to prepare the solid phase IgG Binding ratios have been used to express the results of RF immunoas says (1 2) and if a similar approach is used here to analyse RF binding (Table 1 values in brackets) it is seen that preparation of the solid phase IgG by method A results in significantly reduced binding ratios as compared to those obtained with the solid phase IgGs prepared by methods B and C Neither the assay sensitivity threshold nor the binding ratios differed if the solid phase IgG prepared by method A was pre dried or left hydrated

Preparation of the solid phase IgG by immunolo gical binding of the IgG to an antigen on the solid phase (method B) always gave the lowest assay

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SMOOTH-MUSCLE ANTIBODIES IN RHEUMATOID ARTHRITIS

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Andersen I Andersen P & Graudal H Smooth muscle antibodies in rheumatoid arthritis Acta path microbiol scand Sect. C 88 131–135 1980

Smooth muscle antibodies (SMA) detected by the indirect immunofluorescence method were found more often in rheumatoid arthritis (RA) patients (15 3%) than in normal controls (7 6%) (0 02 > p > 0 01) The increased frequency was due to IgG antibodies which occurred in 12 2% of RA patients in 4 1% of normal controls and in 7 3% of patients with other arthritic diseases Eight of 9 IgG SMA positive RA sera reacted with F actin and one serum contained non actin antibodies In RA ANA were found in 35 7% glomerular antibodies in 1 0% parietal cell antibodies in 4 1% and mitochondrial antibodies in 2 0% SMA were associated with the occurrence of rheumatoid factors and mitochondrial antibodies (0 02 > p and 2p 0 04 respectively) In the SMA positive group the erythrocyte sedimentation rate was higher and elevated serum alkaline phosphatase values were found more often than in the SMA negative group

Key words Smooth muscle antibodies rheumatoid arthritis indirect immunofluorescence

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Antibodies to smooth muscle proteins (SMA) occur especially in chronic hepatitis (13) where IgG antibodies are found in high titres in untreated cases (16) However SMA have also been demonstrated in low titres in other diseases including rheumatoid arthritis (RA) and in normal persons (1 23) Mitochondrial antibodies (MTA) are detected at a high frequency in primary biliary cirrhosis and at lower frequencies in other liver disorders while they occur very seldom in non liver diseases (2 8 16) Abnormal biochemical tests of liver function have been reported in a high proportion of patients with RA (7) and it is therefore possible that SMA and MTA in RA are related to abnormal biochemical liver parameters

In the present study we have investigated the incidence of SMA MTA and other autoantibodies in RA and compared the findings with those in patients with other arthritic diseases and in normal controls It was also studied whether the occurrence of SMA was related to abnormal biochemical liver tests to the occurrence of rheumatoid factors or other autoantibodies

MATERIALS AND METHODS

Autoantibody Tests

Autoantibodies were demonstrated by indirect immunofluorescence (IIF) as previously described (1) Unfixed cryostat sections 4 µm thick of rat stomach and rat kidney were used as antigens and the following antibodies were recorded Smooth muscle antibodies (SMA) reacting with gastr [illegible]

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prepared by method C did not cause an increase in the assay background, a significant reduction in RF binding ratios was always seen This resulted in a slightly increased assay sensitivity threshold

Together these data suggest that covalent binding of the RF-target IgG to a solid phase should be considered when implementing an RF immunoassay This can be done by attaching the IgG to a protein base on a solid phase, as described above, or by attaching the IgG directly to a solid phase as described by *Maiolini et al* (4) These two choices provide RF assays which are equally sensitive and quantitative in terms of IU of RF/ml, suggesting consideration should be given to properties of the solid phase itself In this regard, a particulate solid phase sych as microcrystalline cellulose (4) would seem to be more difficult to manipulate than the polystyrene beads employed here

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Mrs *Marja Terttu Matikainen* kindly assisted in the purification of the human IgG

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SMOOTH-MUSCLE [illegible] IN RHEUMATOID [illegible]

[illegible]

Rheumatism Research [illegible] Medical [illegible] Denmark

Andersen, I. [illegible] Smooth-muscle antibodies in rheumatoid arthritis. Acta path. microbiol. [illegible]

Smooth-muscle antibodies (SMA) [illegible] immunofluorescence method were found more often in rheumatoid arthritis [illegible] than in normal controls [illegible] p > 0.01. The [illegible] which occurred in 12.2 % of RA patients, in 4.1 % of [illegible] with other arthritic diseases. Eight of 9 IgG SMA-positive RA [illegible] and the serum contained non-actin antibodies. In RA ANA were found [illegible] 3 %, parietal cell antibodies in 4.1 % and mitochondrial antibodies [illegible] were associated with the occurrence of rheumatoid [illegible] and mitochondrial antibodies [illegible] 0.3 % respectively. In the SMA positive group the erythrocyte sedimentation rate was higher and elevated serum alkaline phosphatase values were found more often than in the SMA negative group.

Key words: Smooth-muscle antibodies, rheumatoid arthritis, indirect immunofluorescence.

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Antibodies to smooth-muscle proteins (SMA) occur especially in chronic hepatitis (13), where IgG antibodies are found in high titres in untreated cases (16). However, SMA have also been demonstrated in low titres in other diseases, including rheumatoid arthritis (RA) and in normal persons (1, 23). Mitochondrial antibodies (MTA) are detected at a high frequency in primary biliary cirrhosis and at lower frequencies in other liver disorders, while they occur very seldom in non liver diseases (2, 8, 16). Abnormal biochemical tests of liver function have been reported in a high proportion of patients with RA (7) and it is therefore possible that SMA and MTA in RA are related to abnormal biochemical liver parameters.

In the present study we have investigated the incidence of SMA, MTA and other autoantibodies in RA and compared the findings with those in patients with other arthritic diseases and in normal controls. It was also studied whether the occurrence of SMA was related to [illegible] tests, to the occurrence of [illegible] other autoantibodies.

MATERIALS AND METHODS

Autoantibody tests

[illegible] antibodies were detected [illegible] smooth muscle antibodies (SMA) reacting with [illegible] smooth muscle, [illegible] antibodies (GA) reacting with renal [illegible] [illegible]

[illegible] parietal cells, antinuclear [illegible] (ANA) [illegible] nuclei of renal tubular cells and parietal cell antibodies

7

(PA) reacting with the cytoplasm of gastric parietal cells The lowest serum dilution investigated was 1 10

Rheumatoid factors were determined by the Rose Waaler reaction (RW) performed at Statens Seruminstitut Copenhagen and by the Latex fixation test (LFT) using a test kit from Behringwerke AG Germany

Absorption Experiments

Nine IgG SMA positive RA sera were absorbed with F actin tropomyosin and skeletal muscle myosin as previously described (5) Skeletal muscle F actin was prepared from acetone dried powder of rabbit back muscle essentially according to *Spudich & Watt* (20) Tropomyosin and myosin preparations were kindly supplied by Dr *J V Small* the Austrian Academy of Sciences Salzburg Austria F actin was used in a concentration of 6–12 mg per ml of serum and myosin and tropomyosin in concentrations of 40–80 mg per ml of serum

Liver Function Tests

Determination of serum alkaline phosphatase (S AP) and serum glutamic oxaloacetic transaminase (S GOT) values were performed by the routine methods of the department of Clinical Chemistry Aarhus Municipal Hospital Denmark

Subjects

One serum sample from each of 168 patients admitted to the Rheumatology Research Unit Aarhus Municipal Hospital and 629 controls was investigated for autoantibodies The subjects were divided into the following groups

1 Definite rheumatoid arthritis (RA) Sixty-one female and 37 male patients aged 14–79 years All patients fulfilled 5 or more of the criteria of RA as proposed by the American Rheumatism Association (19)

2 Possible and probable RA Eight female and 14 male patients aged 17–79 years Eight patients had rheumatoid factor in their serum (RW titre ≥ 10 and/or LFT titre ≥ 20) but they only fulfilled 2–4 of the criteria for RA (19)

3 Palindromic arthritis Two female and 3 male patients aged 34–49 years They had had afebrile attacks of acute arthritis with pain swelling erythema and tenderness Between attacks the joints were normal

4 Juvenile chronic arthritis Six female and 5 male patients aged 8–32 years In all patients the disease had started before the age of 16 years and they had had arthritis in one or more joints for more than 3 months

5 Reactive arthritis Three female and 2 male patients aged 20–47 years They had developed aseptic arthritis following infection with streptococci (2 pts) *Yersinia enterocolitica* (2 pts) and Chlamydia (1 pt)

6 *Seronegative spondarthritis* Four female and 21 male patients aged 17–75 years Seven had ankylosing spondylitis with low backache stiffness of the back and radiological signs of bilateral sacroiliitis Fifteen had

7 *Controls* This group comprised 301 women and

TABLE 1 *The Occurrence of Antibodies in Rheumatoid Arthritis and other Disorders of Joints*

Disease	Number	Age (years)	% with antibodies							
			RW	LFT	ANA	SMA	GA	CA	MTA	PA
Rheumatoid arthritis	98	14–79	76 5	65 3	35 7	15 3	1 0	0	2 0	4 1
Possible rheum arthr	[illegible]	17 79	36 4	22 7	4 5	4 5	0	4 5	0	0
Palindromic arthritis	5	34–49	40 0	60 0	40 0	20 0	0	0	0	0
Juvenile chr arthritis	11	8–32	9 1	0	9 1	9 1	0	0	0	0
Reactive arthritis	5	20–47	20 0	0	0	20 0	0	0	0	0
Seronegative spondarthritis	25	17–75	0	0	4 0	12 0	4 0	0	0	0
Controls	629	1/–91	N D	N D	7 0	7 6	3 0	0 2	0 2	4 6

328 men aged 5 months – 91 years and the autoantibody findings in this group have been published in detail earlier (2). The group consisted of 406 healthy blood donors, laboratory technicians and medical students aged 18–68 years and 223 hospitalized patients aged 5 months – 91 years. The patients did not have autoimmune, infectious or hepatic diseases.

RESULTS

Autoantibodies

ANA occurred significantly more often in patients with RA (35.7%) than in controls (7.0%) (Table 1, χ^2 test, $0.001 > P$). The incidence of both IgG and IgM ANA was higher in RA than in controls, whereas the incidence in other joint disorders was in the same range as in controls. ANA titres in RA were 10–2560 and they occurred with equal frequency in female (36.1%) and male (35.1%) patients.

LFT titres ≥ 20 were found significantly more often in ANA positive (80.0%) than in ANA negative (57.1%) patients with RA (χ^2 test, $0.05 > p > 0.02$).

The incidence of SMA was significantly higher in RA (15.3%) than in controls (7.6%) (Table 1, χ^2-test, $0.02 > p > 0.01$) and the increased frequency was due to IgG antibodies (χ^2 test, $0.001 > p$), whereas IgM SMA were detected with equal prevalence in RA and controls (Table 2). SMA were found in 18.0% of female and in 10.8% of male patients with RA (not significant). The titres were 10–80, which was the same range as in controls. MTA occurred in 2% of RA patients and in 0.2% of controls ($2p = 0.09$). The incidence of other autoantibodies was similar in patients and controls.

SMA – Rel

of

TABLE 2 *SMA and ANA of IgG and IgM Class in Rheumatoid Arthritis, other Joint Disorders and Controls*

	% with antibodies			
	SMA		ANA	
	IgG	IgM	IgG	IgM
Rheumatoid arthritis (98)	12.2	3.1	24.5	25.5
Other joint disorders (68)	7.3	2.9	4.4	4.4
Controls (629)	4.1	4.5	1.6	5.9

patients (Table 3, χ^2 test, $0.02 > p > 0.01$). LFT-titres ≥ 20 also occurred more often in SMA positive than in SMA negative patients, but this difference was not significant. IgG-GA in a titre of 40 occurred in one RA serum with an IgG SMA titre of 80. No correlation between SMA and ANA could be established. IgG MTA titres of 10 and 20 were found in 2 SMA positive RA patients ($2p = 0.044$). One of the patients also had elevated S-AP.

The mean erythrocyte sedimentation rate (ESR) was higher in SMA positive (mean 57.5 mm/h) than in SMA negative (mean 40.3 mm/h) RA patients (Mann-Whitney test, $p = 0.06$). Elevated S-AP were found in 43 (43.9%) and increased S-GOT in 4 (4.1%) of 98 RA patients. SMA were detected in 18.6% of the patients with elevated and in 12.7% of patients with normal S-AP (not significant). Only one of 4 patients with increased S-GOT had SMA in serum. Thus no correlation between abnormal biochemical liver tests and SMA could be established.

Subcutaneous rheumatic nodules were found in

TABLE 3 *Serological, Biochemical and Clinical Findings in 15 SMA positive and 83 SMA negative Patients with Rheumatoid Arthritis*

	RW pos titre ≥ 10	LFT pos titre ≥ 20	ANA pos	MTA pos	PA pos	GA pos	elevated S-AP	elevated S-GOT	ESR mean mm/h	nodules	duration of disease ≥ 5 yrs
A pos 15	15 (100%)	12 (80%)	7 (46.7%)	2 (13.3%)	1 (6.7%)	1 (6.7%)	8 (53.3%)	1 (6.7%)	57.5	4 (26.7%)	10 (66.7%)
A neg 83	60 (72.3%)	52 (62.7%)	27 (32.5%)	0	3 (3.6%)	0	35 (42.2%)	3 (3.6%)	40.3	15 (18.1%)	38 (45.8%)

Abbreviations, see text

TABLE 4 *IgG-SMA and -ANA Titres before and after Absorption with Muscle Proteins*

Serum	Unabsorbed		Absorption with F-actin		Absorption with Myosin		Absorption with Tropomyosin	
	SMA	ANA	SMA	ANA	SMA	ANA	SMA	ANA
x-60	10	40	<5	40	20	40	10	40
x-61	20	40	<5	40	20	40	20	40
x-63	10		<5		5		5	
x-87	10		<5		n d		5	
x-186	10		<5		5		20	
x-307	80		<10		n d		n d	
x-329	10		<5		5		10	
x-339	40		<10		n d		n d	
x-357	40	20	40	40	40	40	n d	n d

26 7% of the SMA-positive and in 18 1% of the SMA-negative patients (not significant) No correlation between the duration of symptoms and the occurrence of SMA was found

None of the patients with juvenile chronic arthritis or seronegative spondarthritis had iridocyclitis, and no correlation between this disorder and the occurrence of autoantibodies could therefore be demonstrated

Specificity of SMA

Absorption of 8 of 9 IgG-SMA-positive RA sera with F-actin resulted in abolition of the reactions with smooth muscle Absorption with myosin and tropomyosin did not cause a significant decrease in SMA-titres (Table 4) The SMA-titre of serum x-357 was not decreased after absorption with F-actin, myosin or tropomyosin, but the IIF staining was weaker after absorption with F-actin than in the unabsorbed sample Three sera also contained IgG-ANA, but the antibody titres were not affected by the absorption with F-actin, myosin or tropomyosin

DISCUSSION

An increased incidence of SMA has previously been demonstrated in RA by some authors (8, 23) but not by others (9) In the present report, the titres were in the same range in patients and controls, and it was found that the increased frequency of SMA was due to IgG antibodies, whereas IgM-SMA occurred with the same incidence as in controls Similar to what was demonstrated in controls, the occurrence of IgG-SMA was not related to age (1) The present absorption experiments indicate that the majority of IgG-SMA in RA react with F-actin but also that non-actin antibodies may occur The absorption effect could not be due to non immunological binding of immunoglobulins, as the ANA-titres were unaffected SMA in chronic hepatitis also react with F-actin, but in other diseases SMA with other specificities may occur (5)

An increased prevalence of SMA could not be demonstrated in other types of arthritis but this might be due to the low number of patients in these groups The incidence of ANA in RA and other joint disorders was similar to what has been found by others (10, 18, 23)

SMA occur transiently in some infections e g acute hepatitis, infectious mononucleosis, cytomegalovirus infection, measles and mycoplasma pneumoniae infection (3, 4, 6, 11, 12) An association between infections and various arthritides has been suggested (14), and it is possible that SMA in RA may arise after infection However, in infectious diseases SMA are predominantly of the IgM-class whereas in RA they were of the IgG-class High titres of IgG-SMA and measles virus antibodies were, however, found in synovial fluid and serum from a patient with atypical RA (21) This is of interest since some IgG-SMA reacted with actin (5) which has been found in paramyxoviruses (22) Immunization to measles virus might be associated with formation of both specific viral antibodies and actin antibodies It should also be mentioned that ANA and rheumatoid factors have been demonstrated in infectious diseases (15 24) and that SMA occurred most often in rheumatoid factor-positive RA patients

SMA have previously been demonstrated in liver disorders (11, 13, 16), and it is possible that SMA in RA is related to hepatic involvement *Webb et al*

(23) investigated more than two hundred patients and found a higher prevalence of abnormal liver function tests in SMA positive than in SMA negative patients. They (23) also found MTA in 7 (12%) RA patients and 2 of these were SMA positive. In accordance with this we found MTA in 2 (2%) patients who were both SMA positive. Raised alkaline phosphatase values presumably of hepatic origin in a high percentage of RA patients

Rahi et al (17) reported an increased incidence of SMA in uveitis which is associated with e.g. ankylosing spondylitis. In seronegative spondarthritis we could not demonstrate any elevated prevalence of SMA. This might be related to the fact that none of our patients had iridocyclitis.

In RA the SMA positive group was characterized by a higher incidence of rheumatoid factors and elevated alkaline phosphatase values and higher ESR than the SMA negative group. This might be explained by a more severe or more active disease in patients with SMA than in those without. However in order to investigate this it would be necessary to perform a longitudinel study of a larger group of patients.

This work was supported by a grant from *P. Carl Petersens Fond*.

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TABLE 4 *IgG-SMA and -ANA Titres before and after Absorption with Muscle Proteins*

Serum	Unabsorbed		Absorption with F-actin		Myosin		Tropomyosin	
	SMA	ANA	SMA	ANA	SMA	ANA	SMA	ANA
x-60	10	40	<5	40	20	40	10	40
x 61	20	40	<5	40	20	40	20	40
x-63	10		<5		5		5	
x-87	10		<5		n d		5	
x-186	10		<5		5		20	
x-307	80		<10		n d		n d	
x-329	10		<5		5		10	
x-339	40		<10		n d		n d	
x-357	40	20	40	40	40	40	n d	n d

26 7% of the SMA-positive and in 18 1% of the SMA-negative patients (not significant) No correlation between the duration of symptoms and the occurrence of SMA was found

None of the patients with juvenile chronic arthritis or seronegative spondarthritis had iridocyclitis, and no correlation between this disorder and the occurrence of autoantibodies could therefore be demonstrated

Specificity of SMA

Absorption of 8 of 9 IgG-SMA-positive RA sera with F-actin resulted in abolition of the reactions with smooth muscle Absorption with myosin and tropomyosin did not cause a significant decrease in SMA-titres (Table 4) The SMA-titre of serum x-357 was not decreased after absorption with F-actin, myosin or tropomyosin, but the IIF staining was weaker after absorption with F-actin than in the unabsorbed sample Three sera also contained IgG-ANA, but the antibody titres were not affected by the absorption with F-actin myosin or tropomyosin

DISCUSSION

An increased incidence of SMA has previously been demonstrated in RA by some authors (8, 23) but not by others (9) In the present report, the titres were in the same range in patients and controls, and it was found that the increased frequency of SMA was due to IgG antibodies whereas IgM-SMA occurred with the same incidence as in controls Similar to what was demonstrated in controls, the occurrence of IgG-SMA was not related to age (1) The present absorption experiments indicate that the majority of IgG-SMA in RA react with F-actin but also that non-actin antibodies may occur The absorption effect could not be due to non immunological binding of immunoglobulins as the ANA-titres were unaffected SMA in chronic hepatitis also react with F-actin, but in other diseases SMA with other specificities may occur (5)

An increased prevalence of SMA could not be demonstrated in other types of arthritis but this might be due to the low number of patients in these groups The incidence of ANA in RA and other joint disorders was similar to what has been found by others (10, 18, 23)

SMA occur transiently in some infections e g acute hepatitis, infectious mononucleosis cytomegalovirus infection, measles and mycoplasma pneumoniae infection (3, 4, 6, 11, 12) An association between infections and various arthritides has been suggested (14), and it is possible that SMA in RA may arise after infection However, in infectious diseases SMA are predominantly of the IgM class whereas in RA they were of the IgG-class High titres of IgG-SMA and measles virus antibodies were, however, found in synovial fluid and serum from a patient with atypical RA (21) This is of interest since some IgG SMA reacted with actin (5) which has been found in paramyxoviruses (22) Immunization to measles virus might be associated with formation of both specific viral antibodies and actin antibodies It should also be mentioned that ANA and rheumatoid factors have been demonstrated in infectious diseases (15, 24), and that SMA occurred most often in rheumatoid factor-positive RA patients

SMA have previously been demonstrated in liver disorders (11, 13 16) and it is possible that SMA in RA is related to hepatic involvement *Webb et al*

(23) investigated more than two hundred patients and found a higher prevalence of abnormal liver [illegible]tion tests in SMA-positive than in SMA [illegible]tive patients. They (23) also found MTA in 7 [illegible]%) RA patients and 2 of these were SMA [illegible]tive. In accordance with this we found MTA in [illegible]%) patients who were both SMA positive [illegible]sed alkaline phosphatase values presumably of [illegible]atic origin in a high percentage of RA patients [illegible]e been demonstrated previously (7) but we were [illegible]able to show that the presence of SMA could be [illegible]ted to abnormal biochemical liver tests.

Rahi et al (17) reported an increased incidence of [illegible]A in uveitis which is associated with e.g. [illegible]kylosing spondylitis. In seronegative spondarthri[illegible] we could not demonstrate any elevated preva[illegible]ce of SMA. This might be related to the fact that [illegible]ne of our patients had iridocyclitis.

In RA the SMA positive group was characterized [illegible] a higher incidence of rheumatoid factors and [illegible]vated alkaline phosphatase values and higher [illegible]SR than the SMA negative group. This might be [illegible]plained by a more severe or more active disease in [illegible]tients with SMA than in those without. However [illegible] order to investigate this it would be necessary to [illegible]rform a longitudinal study of a larger group of [illegible]tients.

[illegible]his work was supported by a grant from *P. Carl [illegible]etersens Fond*.

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MYCOPLASMA PNEUMONIAE ANTIGENS DEMONSTRATED BY CROSSED IMMUNOELECTROPHORESIS

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Schutten H J Mogensen H H & Lind K *Mycoplasma pneumoniae* antigens demonstrated by crossed immunoelectrophoresis Acta path microbiol scand Sect C 88 137–143 1980

Soluble *Mycoplasma pneumoniae* antigens were analysed by crossed immunoelectrophoresis At least 15 precipitation arcs were developed between the soluble antigens and rabbit anti *Mycoplasma pneumoniae* hyperimmune sera Sera from *Mycoplasma pneumoniae* infected patients formed precipitation arcs which were identified with those formed by a chloroform methanol extract of *Mycoplasma pneumoniae* Low concentrations of human antibodies not detectable in a complement fixation test were found to react with these antigens The antigens were shown to cross react with antigens of *Mycoplasma neurolyticum* Emphasis was placed on the technical development of the crossed immunoelectrophoresis for the analysis of immune precipitable antigens of *Mycoplasma pneumoniae*

Key words Mycoplasma pneumoniae crossed immunoelectrophoresis

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Crossed immunoelectrophoresis (CIE) has been employed in the analysis of antigens of various *Mycoplasma* species (1 6 7) In the present study this technique was used in preliminary investigations of *Mycoplasma pneumoniae* (MP) antigens and their corresponding antibodies in hyperimmune rabbit sera and in patient sera The study was mainly devoted to solving technical problems and to achieving optimal precipitation patterns

MATERIAL AND METHODS

Crossed immunoelectrophoresis The CIE technique of *Laurell* (4) modified by *Axelsen et al* (2) was used Electrophoresis was performed in 1 per cent agarose (LITEX ...

... μl of antigen preparation was applied to wells or slits in the gel The first dimension electrophoresis was performed at 10 V/cm for 50–60 minutes The second dimension electrophoresis was conducted on either 5 × 5 7 × 7 or 10 × 10 cm glass plates for 18 hours at 1–2 V/cm In some experiments an intermediate gel was cast between first and second dimension gels To demonstrate γ mobile antigens the first dimension gel was placed in the middle of the plate with the antigen well in the center and antibody containing gel cast on both sides of it The gels were pressed washed dried and stained with Coomassie Brilliant Blue

Antigens Soluble antigens were prepared from the Mac strain of MP grown in Hayflick s standard broth with HEPES buffer in Roux flasks The glass adherent colonies were washed scraped off and washed again (5) A suspension of these organisms in 0 15 M NaCl ...

dialysed against isotonic phosphate buffer pH 7 4 Some of these preparations were further purified by gel filtration In the isolated "7S fractions only IgG was detectable by CIE using an antiserum to human serum --oteins (Dakopatts Copenhagen)

The DEAE cellulose column was washed with 0 3 M osphate buffer pH 8 0 and the eluted proteins were ecip tated with 25 g ammonium sulphate per 100 ml zate The precipitate was redissolved in H_2O and ssed through an Ultrogel AcA 34 (LKB) column actions shown to contain IgM by fused rocket imunoelectrophoresis (2) with antiserum to human μ ains (Dakopatts) were pooled For further purification column of rabbit IgG to human γ chains (Dakopatts) oupled to CNBr Sepharose 4B (Pharmacia) was used to emove aggregated IgG The eluted proteins which onstituted the "19S fractions" contained no detectable gG

Detergent The non ionic detergent Triton X 100 was sed

Trypt c d gest on The CM extract of MP was ncubated with trypsin (Trypure Novo) at a concentra ion of about 1 mg/ml at 37 °C for 60 m nutes pH 8 0) gestion was stopped by Trasylol (Bayer)

RESULTS

Precipitation Pattern in CIE

With 25 μl antigen (2–5 mg protein per ml) and 500 μl antibody in the second dimension gel generally 15 precipitation arcs could be identified on 7×7 cm plates most of which were distinct and reproducible (Fig 1a) With some pools of hyperim mune sera up to 24 precipitation arcs were seen No precipitate with γ mobility was observed

Two arcs were identified as growth medium immune precipitates by incorporating the medium in an intermediate gel By this technique an absorption is made *in situ* (Fig 1b) When the antiserum was absorbed with medium prior to electrophoresis the same two arcs disappeared

Variables Influencing the Precipitation Pattern

1) Antigen preparation The time and tempera ture of the sonication were highly critical since deviation from the optimal treatment resulted in loss of precipitation arcs An antigen suspension of 300 μl containing 3–5 mg protein per ml was sonicated for 2×30 seconds in an ice water bath The amplitude was 8 μm Immediately after sonication the antigen preparation was centrifuga ted at 40 000 g for 10 minutes When this antigen was kept at room temperature for more than 30 minutes before application some of the arcs would not appear

2) Antibody preparation Optimal precipitation patterns were achieved by pooling selected rabbit hyperimmune sera Differences between antibody preparations raised with solubilized and non solubilized MP antigens were mainly of quantitative nature

3) Effect of detergent Triton X 100 was effective in solubilizing MP as judged from protein determi nation However the gain in precipitation arcs was doubtful whether Triton was added to the antigen or to the gel hence this detergent was omitted in the experiments presented

Antibody Reactivity to CM extracted Antigens

When a CM extract of MP was run in the first dimension two precipitation arcs were formed with rabbit hyperimmune serum These arcs could be shown to have antigenic identity with precipitation arcs formed between whole antigen and rabbit hyperimmune serum by incorporation of the CM extract in an intermediate gel (Fig 1c and d) This "CM precipitate" which stained with Sudan Black consistently showed the same configuration with a confluence of two arcs whether formed by the whole antigen preparation or by the CM extract alone When the whole antigen was run against an IgG preparation of a pool of anti MP CF positive human sera only the "CM precipitate" was formed (Fig 1e) Again this could be identified by a CM extract of MP incorporated in an intermediate gel (Fig 1f)

Human sera were tested for precipitating antibo-dies by incorporating them in intermediate gels between the whole antigen and the rabbit hyperim mune serum In this position human anti MP antibodies would be expected to reduce the area of some precipitation arcs with a reduction proportio-nal to the increase in antibody concentration (2) Figs 2a b c d show the effect of different intermediate gels Only certain CF positive human sera (see Fig 2c) caused a definite depression of certain precipitates as compared to CF negative sera A striking finding was the significant depres sion of the CM precipitate" caused by CF non reactive sera (Fig 2b) The same effect was observed with rabbit and guinea pig sera in the intermediate gel whether CF positive or non reactive The serum components responsible for this depressing effect were tentatively investigated Figs 3a b c d e show the effect of different intermediate gels containing a) saline (no depression) b) a 7S fraction and c) a 19S fraction from a pool of CF positive sera (both depression) d) a 7S fraction of a CF non reactive pool (depression) and e) a 19S fraction of the same pool (no depression) The depression seen in c) and d) was abolished by

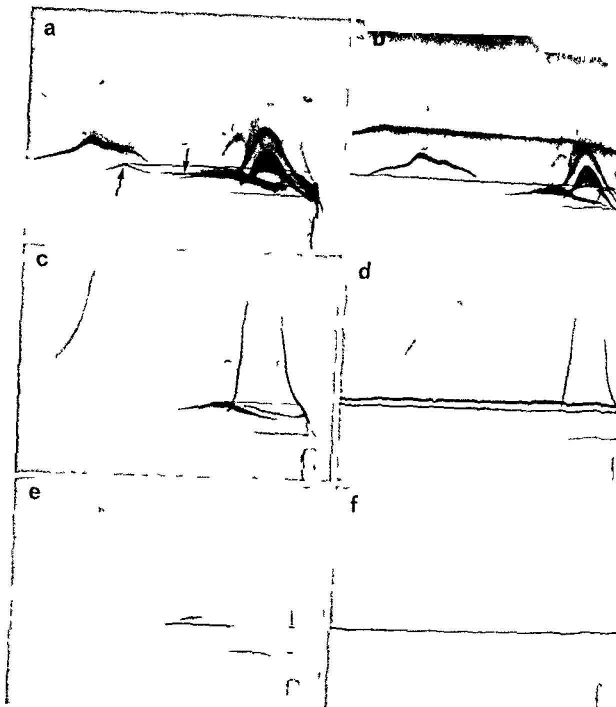


Fig 1 Effect of absorption *in situ* with growth medium and CM extract of MP a) and b) first dimension 25 µl MP sonicate Top gels 500 µl hyperimmune anti MP serum Intermediate gels a) saline b) growth medium *Arrows* indicate growth medium precipitates c) and d) first dimension 25 µl MP sonicate Top gels 200 µl hyperimmune anti MP serum Intermediate gels c) saline d) CM extract of MP e) and f) first dimension 25 µl MP sonicate Second dimension IgG preparation of human anti MP sera Intermediate gels e) saline f) CM extract of MP

Freund s incomplete adjuvant Another group of rabbits was injected with sonicated *MP solubilized* by L α lysophosphatidyl choline (about 0 2 per cent W/V) following the same schedule Initially these rabbits responded with very high antibody *titres which* gradually fell to the level achieved in the rabbits immunized *with non solubilized MP The complement* fixing (CF) antibody titre (5) in sera to be pooled was ≧ 1000

Partial purification of the γ globulin fraction was performed by either ammonium sulphate precipitation DEAE cellulose chromatography or gel filtration

Purification of human γ globulins Pools of human sera were dialysed against 0 01 M phosphate buffer pH 8 0 and passed through a DEAE cellulose column equilibrated with the same buffer The first peak eluted using the equilibration buffer was concentrated and

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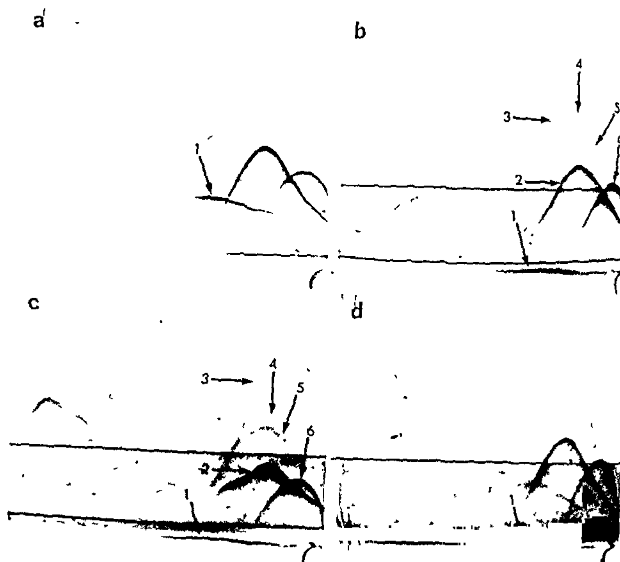


Fig 2 Effect of human sera in the intermediate gels First dimension 10 µl MP sonicate *Top gels* 200 µl hyperimmune anti-MP serum Intermediate gels a) saline b) anti-MP CF-negative human serum c) and d) anti MP CF-positive sera with titres of 256 and ≧ 4000 respectively

absorption with whole MP organisms After dilution of the 7S fraction of the CF-positive pool used in b) to low CF titre, the depressing effect was still present After absorption with whole MP cells this effect was eliminated, and the antibodies were no longer detectable However, even after repeated absorptions of the undiluted, strongly positive 7S fraction, this absorbate still caused the depression of the "CM precipitate", when placed in the intermediate gel as shown in b) The titre of the absorbate could not be evaluated by the CF test because of anticomplementary effect, probably due to immune complexes All fractions were adjusted to contain IgG and IgM corresponding to the content of 250 µl normal human serum by rocket immunoelectrophoresis (2) These results suggest that the CF test was too insensitive to detect the low concentrations of antibodies to MP responsible for the depressing effect

Cross-reactivity between Mycoplasma (M) *Species Studied by Absorption with Antigen*

A CM extract of *M neurolyticum* was found to react with human anti-MP sera in the CF test, and the antibody titres were the same as obtained with an MP CM extract Both antigens could be used at a dilution of 1 100 In CIE the CM extract of *M neurolyticum* formed a precipitate with rabbit anti MP sera This precipitate was totally abolished by inserting an intermediate gel containing CM extracted MP antigens On the other hand, the precipitate formed between the MP CM antigen and the same antisera was only weakened by the *M neurolyticum* CM antigen in an intermediate gel This suggests a

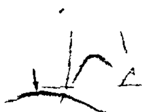
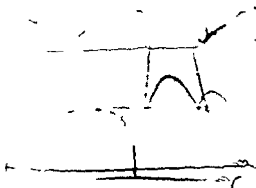
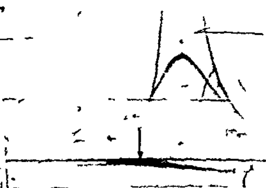
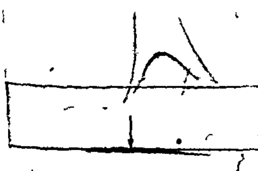
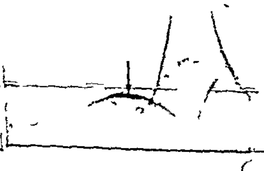


Fig 3 Effect of different human γ-globulin fractions in the intermediate gels First dimension 10 µl MP sonicate Top gels 200 µl hyperimmune anti MP sera Intermediate gels a) saline b) 7S fraction and c) 19S fraction of human anti MP CF positive sera d) 7S fraction and e) 19S fraction of human anti MP CF non reactive sera









partial antigenic identity between lipid antigens of the two *Mycoplasma* species

Tryptic digestion of the CM extract of MP did not affect the ... of the antigen or of anti MP human sera when this antigen was used in a CF test The precipitation pattern of this digest was only slightly altered showing a more pronounced separation of the two arcs characteristic of the "CM precipitate"

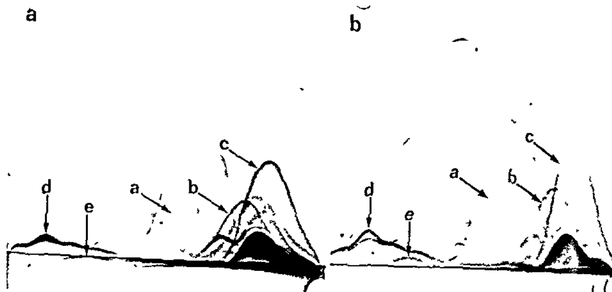


Fig 4 Effect of absorption with MP organisms First dimension 10 µl MP sonicate Top gels 500 µl anti M hyperimmune sera a) unabsorbed b) absorbed with whole MP cells

Absorption of hyperimmune sera with whole, washed MP cells showed detectable increase in the area of some of the precipitation arcs, while others were unchanged (Fig 4) The elevation of particular precipitates is assumed to be due to absorption of antibodies in the antiserum by antigens on the surface of the cells After absorption of hyperimmune serum to MP with whole cells of *M hominis* no changes in peak heights were observed, except for the precipitates representing growth medium factors, which were eliminated A soluble antigen, prepared from *M hominis* as described for MP, was placed in an intermediate gel between soluble MP antigens and rabbit anti-MP serum in the CIE No cross-reactions were observed

DISCUSSION

The CIE offers a means of analysing immunochemically such antigens of MP that are precipitable by antibodies from hyperimmunized animals The versatility of the test depends on the solubility of the antigens and their electrical charge, as well as on the ability of the animals to form precipitating antibodies Although antigens of MP seem to be less readily precipitated by hyperimmune sera than antigens of other *Mycoplasma* species (1, 6, 7) a maximum of 24 different antigens could be demonstrated by the CIE

MP antibodies in human sera that are detected in high titres by the CF test give rise only to the "CM precipitate" However, when human anti MP positive sera were placed in an intermediate gel betwee the sonicated MP antigen and hyperimmune rabbi sera, some additional precipitates were depresse owing to a contribution of human antibodies Eve low concentrations of MP antibodies, not detectabl in the CF test, would exert a depressing effect on the "CM precipitate" when placed in the gel between the sonicated MP and hyperimmune sera It is not known whether these antibodies were elicited during previous MP infection or by cross reacting antigens

Kenny (3) has shown that CM-extracted lipids from *M neurolyticum* and from MP cross-react in the CF test The fact that CM-extracted lipids from MP and *M neurolyticum* did cross-react in both the CF and the CIE tests indicates the presence of common CF and precipitating antigens in the two microorganisms Although CIE may be useful in the analysis of antigens which are active in natural infection, it seems to be of little clinical diagnostic value The method has its main potentialities in the analysis of immunological cross-reactions

We thank *Hanne Mandrup Møller* for skilful and meticulous technical assistance

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SPECIFIC DETECTION OF DIFFERENT PLASMA PROTEINS IN GEL FILTRATION FRACTIONS BY SOLID PHASE ENZYMEIMMUNOASSAY (EIA)

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Viljanen M K Specific detection of different plasma proteins in gel filtration fractions by solid phase enzymeimmunoassay (EIA) Acta path microbiol scand Sect C 88 145–147 1980

An enzymeimmunoassay (EIA) is described enabling sensitive and specific immunodetection of different proteins in analytical and preparative fractionation of biological mixtures The method is based on the ability of polystyrene to bind proteins and polypeptides Commercially available rabbit antisera are used combined with the use of commercial alkaline phosphatase conjugated anti rabbit IgG antibodies The good assortment of rabbit antisera available makes possible identification of hundreds of proteins in biological samples by the present method

Key words Enzymeimmunoassay gel filtration

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Analytical and preparative molecular sieving of biological protein mixtures usually yields a large number of fractions The identification of different proteins and polypeptides in the fractions can be based on functional or chemical properties of the molecules to be identified e g enzymatic activity dye binding etc However the vast majority of proteins in biological samples lack a suitable marker for detection and they have to be identified by immunochemical methods using specific antisera The immunochemical detection is mostly carried out by different modifications of immunoprecipitation in gel phase (3 4) These methods are however mostly qualitative and reagent-consuming which restricts their use in paralleled identification of several proteins in a large number of fractions

In the present work a method is described for detection of proteins using commercially available specific antisera and the ability of polystyrene to bind proteins (1) The enzymatic indicator reaction used yields a coloured product allowing a visual reading of the results For more precise measurement of e g elution volumes after molecular sieving a photometric reading of optical absorbances can be used The method is applied for the detection of a δ-reactive monocomponent protein in Sephadex G 200 fractions of a myeloma patient s plasma

MATERIALS AND METHODS

Gel filtration Sephadex G 200 column (100 cm × 5 cm²) was used for fractionation of 0 5 ml of normal human plasma or plasma from the myeloma patient (MAL) The routine immunochemical analysis revealed that MAL plasma contained a monocomponent protein carrying δ heavy chain determinants but not κ or λ light chain determinants The clinical and laboratory findings of this first δ heavy chain disease and a detailed characterization of the monocomponent protein will be published elsewhere (5 6) Tris HCL buffer (pH 8 0) at a flow rate 5 ml per hour was used in the elution The fraction volume was 10 ml Total protein concentration of the

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SPECIFIC DETECTION OF DIFFERENT PLASMA PROTEINS IN GEL FILTRATION FRACTIONS BY SOLID PHASE ENZYMEIMMUNOASSAY (EIA)

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Viljanen M K Specific detection of different plasma proteins in gel filtration fractions by solid phase enzymeimmunoassay (EIA) Acta path microbiol scand Sect C *88* 145–147 1980

An enzymeimmunoassay (EIA) is described enabling sensitive and specific immunodetection of different proteins in analytical and preparative fractionation of biological mixtures The method is based on the ability of polystyrene to bind proteins and polypeptides Commercially available rabbit antisera are used combined with the use of commercial alkaline phosphatase conjugated anti rabbit IgG antibodies The good assortment of rabbit antisera available makes possible identification of hundreds of proteins in biological samples by the present method

Key words Enzymeimmunoassay gel filtration

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Analytical and preparative molecular sieving of biological protein mixtures usually yields a large number of fractions The identification of different proteins and polypeptides in the fractions can be based on functional or chemical properties of the molecules to be identified e g enzymatic activity dye binding etc However the vast majority of proteins in biological samples lack a suitable marker for detection and they have to be identified by immunochemical methods using specific antisera The immunochemical detection is mostly carried out by different modifications of immunoprecipitation in gel phase (3 4) These methods are however mostly qualitative and reagent-consuming which restricts their use in paralleled identification of several proteins in a large number of fractions

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MATERIALS AND METHODS

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of the [illegible] will be published elsewhere (5 6) Tris HCL buffer (pH 8 0) at a flow rate 5 ml per hour was used in the elution The fraction volume was 10 ml Total protein concentration of the

(mol wt 900000) When extrapolated from the elution volumes of IgG (γ heavy chains) C3 transferrin and albumin the molecular size of the paraprotein is about 250000 daltons

The maximum protein concentration which does not exceed the binding capacity of the solid phase can be estimated from the top value of the albumin curve This concentration is 2 1 mg/ml and when the optimal dilution (1 100 1000) is taken in account one can estimate that the maximum protein concentration is between 2–20 μg/ml The detection limit of this method can be estimated from the descending arc of the albumin curve Protein concentration of the fraction giving in albumin EIA still significantly higher absorbance than the background value was 0 1 mg/ml When this figure is divided by the optimal dilution for albumin detection (1 1000) the detection limit is 0 1 μg/ml

DISCUSSION

A good assortment of commercial antisera is available for immunological detection of different proteins in analytical and preparative fractionations For the estimation of molecular size of an unknown protein by gel filtration at least 3–5 different markers of known size are needed This necessitates a simultaneous assaying of a relatively large number of samples and a parallel use of different techniques e g isotopic markers enzymatic markers etc It is possible by the present EIA method to measure hundreds of samples daily and to use proteins preexisting in biological fluids as markers and no adding of external markers is needed Photometric reading of absorbances in EIA makes possible a precise estimation of elution volumes This can be achieved mostly by assaying the samples in one dilution only The expensive antisera are used in high dilutions in the assay and their consumption is low

The relatively narrow working range of the assay necessitates adjustment of total protein concentration in each individual case suitable for the binding capacity of the solid phase Owing to competition between different proteins the method cannot detect a protein existing as a very minor component among others of same molecular size Therefore the detection limit 0 1 μg/ml must be considered somewhat theoretical The specificity of the antisera used is out of the scope of this report but it is evident that possible slight cross reactivities do not have any effect on the estimation of the elution volumes of the proteins studied On the other hand EIA provides a sensitive tool to study the specificity of antisera produced against purified proteins

EIA can be applied to all fractionations where the proteins can be eluted out of the medium e g gel electrophoresis (6) The sensitivity of the method is high enough for assays made in micro scale using only microliters of samples The photometric reading of absorbances used in the present work necessitates polystyrene cuvettes of very high optical quality However quite satisfactory results can be obtained using ordinary disposable polystyrene tubes and visual estimation of the intensity of the colour The reaction mixtures can also be transferred to normal photometric cuvettes for measurement of absorbance

The molecular size estimation of the paraprotein by EIA in the present work is in good accordance with the results received by other techniques (6) During the purification and characterization process of the paraprotein in this first δ heavy chain disease EIA proved to be valuable

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I wish to thank Mrs *Maija Salonen* for expert technical assistance

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fractions was estimated by measuring absorption of ultraviolet light (280 nm Uvicord LKB Sweden) A more accurate quantification of proteins was carried out by the method of *Lowry et al* (2)

Performance of EIA The tests for specific protein detection by EIA were carried out in disposable polystyrene nine cuvette blocks (Finnpipette Labsys tems Helsinki Finland) The following proteins were detected in the above mentioned fractions of MAL plasma γ μ and δ heavy chains albumin C3 component of the complement system and transferrin In order to clarify the optimal dilution of the fractions they were diluted 1 10 1 100 1 1000 and 1 10000 by 0 05 M PBS pH 7 4 (PBS) All determinations were made in triplicate A volume of 200 μl of each dilution was added to the cuvettes and incubated 3 h at + 37 °C for attachment of the proteins to the solid phase The cuvettes were washed three times with 0 4 ml of PBS The solid phase was saturated by incubating the cuvettes 2 h at + 37 °C with 200 μl of PBS supplemented with 1 0 % of bovine serum albumin (BSA PBS) The cuvettes were washed once with 0 4 ml of physiological saline with 0 05 % of Tween 20 (NaCl Tw) Commercially available rabbit antisera against heavy chains C3 albumin and transferrin (Behringwerke AG Frankfurt am Main Germany) were diluted 1 1000 with BSA PBS and added to the cuvettes in a volume of 150 μl The cuvettes were incubated 1 h at + 37 °C and washed three times with 0 4 ml of NaCl Tw 150 μl of alkaline phosphatase conjugated swine anti rabbit IgG (Orion Diagnostica Helsinki Finland) diluted 1 200 with BSA PBS was added to the cuvettes The cuvettes were incubated 4 h at + 37 °C and washed as previously The amount of alkaline phosphatase bound to the cuvettes was quantified using p nitrophenylphosphate (Sigma Chemical Co St Louis Mo USA) as a substrate in a concentration of 2 mg/ml in glycine $MgCl_2$ buffer pH 10 0 (Orion Diagnostica) The intensity of the colour of the product was estimated visually or its absorbance was measured at a wavelength of 405 nm in a nine channel photometer with a vertical beam specifically adapted for the nine cuvette blocks (Finnpipette Analyzer System FP 9 Finnpipette Labsystems)

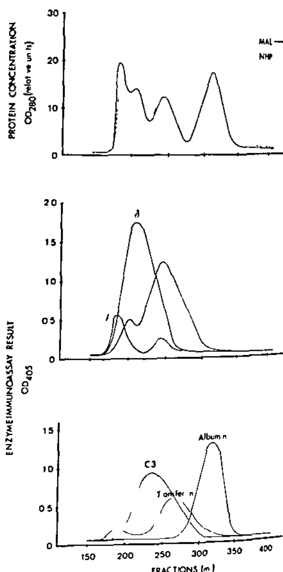


Fig 1 Sephadex G 200 fractionation of 0 5 ml of normal human plasma (NHP) or plasma from a myeloma patient (MAL) having δ reactive monocomponent Comparison between total protein concentration curve and enzymeimmunoassay (EIA) detection patterns of six different serum proteins in MAL plasma (γ μ and δ heavy chains C3 component of complement transferrin and albumin)

RESULTS

A clear prezone phenomenon could be observed in the effect of total protein concentration on the binding of proteins to the solid phase The optimal dilution of fractions for albumin detection was 1 1000 In lower dilutions the albumin peak was irregular in shape and standard deviation between parallel determinations increased Heavy chains could be detected in both dilutions 1 1000 and 1 100 In 1 10 also the heavy chain peaks became irregular and no exact elution volumes for these proteins could be detected The optimal dilution for C3 and transferrin was 1 100 Possibly owing to a competition by other proteins existing in the fractions the binding of C3 and transferrin in 1 10 was almost negligible In higher dilutions than 1 1000 the concentration of these proteins was evidently so low that the absorbances did not significantly exceed the background value (= OD_{405}0 100) In the above mentioned optimal dilutions the standard deviation of triplicate determinations varied from 6 to 20 per cent of the mean values

The detection patterns made from the fractions of MAL plasma are depicted in Fig 1 The paraprotein is located between C3 (mol wt 185 000) and IgM

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ANTIBODY RESPONSE IN PATIENTS WITH *PSEUDOMONAS AERUGINOSA* INFECTION TO A 'COMMON ANTIGEN' FROM *P. AERUGINOSA* ANALYSED BY MEANS OF QUANTITATIVE IMMUNOELECTROPHORETIC METHODS

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Høiby N Hertz J B & Sompolinsky D Antibody response in patients with *Pseudomonas aeruginosa* infection to a common antigen of *P aeruginosa* analysed by means of quantitive immunoelectrophoretic methods Acta path microbiol scand Sect. C *88* 149–154 1980

381 sera from 101 patients suffering from *Pseudomonas aeruginosa* infection have been investigated for antibodies against a common antigen (CA) and other antigens from *P aeruginosa* by means of crossed immunoelectrophoresis with intermediate gel using a polyspecific *P aeruginosa* antigen/antibody reference system The earliest antibody response d [illegible] CA antibodies in only 38% of the patient [illegible] increased CA antibodies in 71% of the p [illegible] found that the antibody response to CA [illegible] the CA molecule and the *Pseudomonas*-specific part with individual variations During infections caused by other bacteria containing CA one third of the patients developed antibodies against CA The diagnostic implications of the results are discussed

Key words *Pseudomonas aeruginosa* common antigen cross reactions antibody response immunoelectrophoresis

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Quantitative immunoelectrophoretic methods have revealed a complex antigenic structure of *Pseudomonas aeruginosa* 64 different antigens have been described in this species ten of which cross react with other bacterial species (3 7) One of the antigens (No 10 in the reference system (3) designated common antigen (CA) from *P aeruginosa*) has been characterized further This is a protein which is present in a wide range of aerobic and facultative anaerobic gram negative bacteria (3 11 12) On the basis of analysis by crossed line immunoelectrophoresis (absorption *in situ* of antibodies) CA gives 100% cross-reaction with the corresponding antigen in a number of other *Pseudomonas* species whereas the degree of cross reactivity is less pronounced with the corresponding antigen in species belonging to other genera (3) Accordingly it has been proposed that the CA molecule contains both cross reactive antigenic determinants and determinants which are specific for *P aeruginosa* and a few other *Pseudomonas* species (3) The distribution of CA in other bacterial species has been confirmed by means of similar immunoelectrophoretic methods using reference systems of *Bordetella pertussis Neisseria meningitidis* and *Haemophilus influenzae* (1, 5 10)

Normally-occurring antibodies against CA have been found in sera from 54% of normal persons, but the 95% range of titres was only 0–2 (4) These normally-occurring antibodies were found to be directed against the cross reactive antigenic determi-

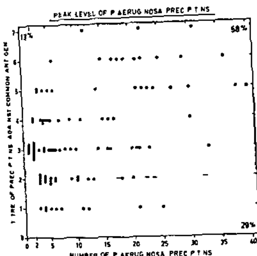


Fig 2 Highest number of *P aeruginosa* precipitins (abscissa) and corresponding titre of precipitins against CA (ordinate) detected in sera from the 101 patients harbouring *P aeruginosa* who were investigated repeatedly during the infection Each of the patients is represented by one filled circle one serum Broken lines indicate the upper normal limits of precipitins and titre of CA antibodies

tins *and/or* ≧ titre 3 of CA antibodies) (4) was detected

From 50 of the patients (27 with cystic fibrosis and 23 suffering from other diseases) sera (2–6) were available before and during the initial rise of antibodies against *P aeruginosa* The results obtained with the serum sample from each patient which showed the earliest antibody response (increased CA antibody titre *and/or* increased number of precipitins) are shown in Fig 1 In most cases (62%) the total number of precipitins rose above normal values before the titre of CA antibodies rose but in 24% of the cases the situation was *vice versa* (Fig 1) In 14% of the cases there was an initial rise in both the total number of precipitins and in the titre of CA antibodies

Fig 2 shows the highest measured level of *P aeruginosa* precipitins in sera from the 101 patients Most of the patients who developed an antibody response mounting above normal values showed both an increased total number of precipitins and an increased titre of CA antibodies (58%) However 29% of the patients showed only an increased total number of precipitins (up to 25) and 13% only an increased titre of CA antibodies Two examples of the development of precipitins against *P aeruginosa* antigens are given in Fig 3





Fig 3 Antibodies against CA and other *P aeruginosa* antigens in sera from patients harbouring *P aeruginosa* determined by means of crossed immunoelectrophoresis with intermediate gel Technical 2 µl *P aeruginosa* St Ag in the wells first dimension electrophoresis anode to the right second dimension electrophoresis anode at the top Second dimension reference gels contain rabbit antiserum against *P aeruginosa* (St Ab) (20µl/cm²) Staining Coomassie brilliant blue

A The intermediate gel contains serum (40 µl/cm²) from a patient suffering from chronic *P aeruginosa* lung infection The patients produced detectable antibodies against 25 of the *P aeruginosa* antigens including CA antibodies (arrow) of titre 1 (inward feet reaction)

B The intermediate gel contains serum (40µl/cm²) from another patient suffering from chronic *P aeruginosa* lung infection The patient produced detectable antibodies against 15 of the *P aeruginosa* antigens including CA antibodies (arrow) of titre 6

Specificity of Antibodies against CA in Serum from Patients Harbouring P aeruginosa

The results of the absorption *in situ* of antibodies against CA in sera from 24 patients are shown in

nants of CA, and it is possible that these 'natural' antibodies are induced by the normal mucosal flora (1, 4, 5)

Cross-reactive antigens are potential tools in diagnosis and prophylaxis of bacterial infections, and information regarding the antibody response against such antigens is required The aim of the present work was therefore to compare the antibody response to CA in patients during infection caused by *P aeruginosa* with the antibody response to other *P aeruginosa* antigens, and to determine the specificity of antibodies to CA Furthermore, in a few cases the antibody response to CA was also investigated in patients with infections caused by other bacteria

MATERIAL AND METHODS

Patients

381 sera were obtained from 101 patients harbouring *P aeruginosa* 52 of the patients suffered from cystic fibrosis and harboured *P aeruginosa* in the lungs as described previously (8) 49 of the patients suffered from a variety of other diseases and harboured *P aeruginosa* in one or more regions (respiratory tract urinary tract middle ear gall bladder blood and cerebrospinal fluid) as reported in detail previously (6) The duration of the colonization at the time of the highest antibody response measured was known in 81 of the patients (mean duration 1 8 years range 1 week – 7 years)

Thirteen sera were obtained from 13 patients with other infections viz five patients with meningococcal meningitis one with *Escherichia coli* meningitis two with pneumococcal meningitis one with *E coli* septicaemia one with a *Staphylococcus aureus* abscess one with urinary tract infection caused by *E coli* and *Klebsiella pneumoniae* one with urinary tract infection caused by *Streptococcus faecalis* and one with *Pseudomonas stutzeri* (mucoid) lung infection Precipitating antibodies against the offending bacteria were detectable in these sera by crossed immunoelectrophoresis (1–35 precipitins)

Immunoelectrophoretic Methods

Crossed immunoelectrophoresis with intermediate gel and crossed line immunoelectrophoresis were performed as described previously (3 4) In order to detect antibodies in the patients sera 2 μl *P aeruginosa* antigen (St Ag batch 1) was run against patient serum in the intermediate gel (40 μl/cm²) and pooled concentrated rabbit antiserum against *P aeruginosa* (St Ab pool 7–17) in the reference gel (20 μl/cm²) in 5 × 5 cm glass plates A control with 0 154 M NaCl in the intermediate gel was included in the run every day The number of immunoprecipitates visible in plates with human serum was counted (= the number of precipitins) and the precipitating antibodies against CA were semiquantified by comparing the area included by human precipitins with the area of corresponding rabbit precipitins in six standard plates containing St Ab in the intermediate gel

0 < titre 1 ≦ 1 μl/cm² < titre 2 ≦ 5 μl/cm² < titre 3 ≦ 10 μl/cm² < titre 4 ≦ 20 μl/cm² < titre 5 ≦ [illegible] μl/cm² < titre 6 ≦ 80 μl/cm² < titre 7

In order to determine the specificity of the hum[an] antibodies against CA sera from 24 of the patients we[re] investigated further These patients were selected as [a] representative sample of the 101 patients in this stu[dy] (Table 1) 1 μl St Ag was run against each patien[t] serum in the reference gel (10 μl/cm²) and with antige[ns] from *Pseudomonas pseudoalcaligenes E coli Prote[us] mirabilis H influenzae* (non-capsulate) in the interme[di]ate gel (20 μl/cm²) or with *S aureus* without protein [A] and with 0 154 M NaCl as controls The percenta[ge] absorption of antibodies against CA was estimated [by] comparison of the increase in the area of the C[A] immunoprecipitate with a series of standard plat[es] containing St Ab 20 15 10 7 5 5 and 2 5 μl/cm²

All antigen samples used consisted of water solub[le] antigens obtained by sonication The antigens and th[e] method of antigen production have been described i[n] detail previously (2 3)

RESULTS

Development of Precipitating Antibodies against CA and other P aeruginosa *Antigens in Patients Harbouring* P aeruginosa

In 305 (80%) of the 381 sera from the 101 patients an antibody response against *P aeruginosa* which mounted above normal values (≧ 3 precipi[tins]

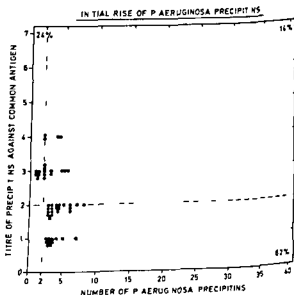


Fig 1 Initial antibody response to *P aeruginosa* antigens mounting above upper normal limits (broken lines) in sera from the 50 patients harbouring *P aeruginosa* who were investigated repeatedly during the infection Each of the patients is represented by one filled circle = one serum Abscissa total number of *P aeruginosa* precipitins Ordinate titre of precipitins against CA

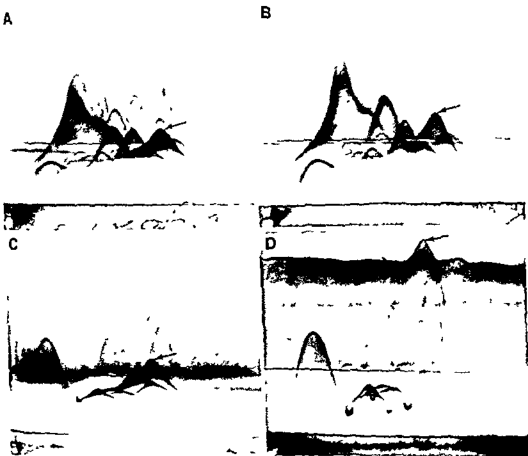


Fig 4 Absorption of antibodies *in situ* by means of crossed line immunoelectrophoresis

A Control plate showing 2 µl *P aeruginosa* antigens (St Ag) in the well run against serum from a patient suffering from chronic *P aeruginosa* lung infection in the second dimension reference gel (10 µl/cm²) 25 precipitating antibodies were demonstrable in the serum including antibodies against CA (arrow) Saline in the intermediate gel

B Same as *A* but with antigens from *E coli* (20 µl/cm²) in the intermediate gel Compared with *A* the area of the CA precipitate (arrow) increased only slightly after absorption of cross reactive antibodies by *E coli* antigens The area of a few other precipitates corresponding to other cross reactive *P aeruginosa* antigens (ref 3) have also increased

[illegible]

CA precipitate (arrows) increased greatly after absorption of cross reactive antibodies by *E coli* antigens The area of a few other precipitates corresponding to other cross reactive *P aeruginosa* antigens (ref 3) have also increased

Conclusion The first patient (A and B) produced predominantly antibodies against the *Pseudomonas* specific part of CA whereas the second patient (C and D) produced predominantly antibodies against the cross-reactive part of CA

reactive part of the *Pseudomonas*-specific part of CA The reason for the varying individual antibody response is not known but genetic differences in the immune response and the immunological tolerance phenomenon may be considered The same explanations may by offered as regards the results obtained with sera from patients suffering from infections caused by bacterial species other than *Pseudomonas* containing CA

According to the results of this work, the measurement of CA antibodies alone is not as reliable a tool in the diagnosis of *P aeruginosa*

TABLE 1 *Absorption of* P aeruginosa-*CA Antibodies in 24 Human Sera*[a] *by Antigens of Five Other Bacterial Speci*

		Origin of antigens and number of sera				
		P pseudoalcaligenes	*E coli*	*P mirabilis*	*H influenzae*	*S aureus*
Percentage	100%	24	7	8	6	
Absorption of	100–75%		2	2		
CA antibodies	75–50%		5	4	3	
	50–25%		7	7	12	
	0%[b]		3	3	3	24

a) *Number of P aeruginosa* antibodies in the 24 sera (range) 1–46 Titres of antibodies against CA (range) 2–7
b) < 25% absorption is considered *insignificant (3)*

Table 1 and examples are given *in Fig 4 In all* cases antigens from *P pseudoalcaligenes* could absorb all precipitating antibodies *against* CA, *whereas S aureus* antigens could not absorb antibodies at all However, *using* antigens from *E coli, P mirabilis* and *H influenzae*, the results obtained with sera from different patients varied Some of the patients produced solely or predominantly antibodies against the cross-reactive part of CA, while others produced predominantly antibodies against the *Pseudomonas*-specific part of CA (Table 1) The degree of absorption of antibodies by these three gram-negative rods was generally similar in the individual sera There was no correlation between the degree of absorption of antibodies against CA and the titre of CA antibodies or the total number of precipitins in the sera

Development of Precipitating Antibodies against CA in Serum from Patients Suffering from other Bacterial Infections

None of the four patients with infections caused by gram-positive cocci developed CA antibodies, whereas three (33%) of the nine patients with infections caused by gram-negative bacteria developed CA antibodies (infections caused by *N meningitidis* and *E coli* titre 3, and by *P stutzeri* titre 5)

DISCUSSION

Common antigen of *P aeruginosa* has recently been purified and characterized as a protein with mol wt 665,000–900,000 consisting of polypeptide subunits of mol wt 60,000 (11, 12) According to *Kayser* (9), it is situated in the inner part of the cell envelope Because of the occurrence of CA in a wide range of predominantly gram-negative bacterial species and the presence of precipitating antibodie against CA in 54% of normal human sera (and als in rabbit sera), the immunological and pathophysio logical roles of this antigen during infection are o interest

The present study shows that the initial antibod response in 62% of the patients does not includ increased titre of CA antibodies (Fig 1) However later in the infection the majority of the patient develop CA antibodies, but even then 29% of th patients with an antibody response against other *P aeruginosa* antigens do not produce increased titr of CA antibodies (Fig 2) 14 out of the 31 patient who did not develop CA antibodies during th initial antibody response (Fig 1) had low titr (normal) CA antibodies before the infection, and this was also the case in 18 of the 29 patients wh did not develop CA antibodies during the highes measured antibody response (Fig 2) In these latte patients, it could have been expected that the *P aeruginosa* infection would have provoked a secon dary 'booster' response of CA antibodies

The individual variation of the antibody respons to CA is also reflected in the absorption *in sit* results obtained with antigens from *E coli P mirabilis* and *H influenzae, i e* some of the patient produced predominantly antibodies against th common cross reactive part of CA, whereas othe patients produced predominantly antibodies against the *Pseudomonas*-specific part of CA (Table 1, Fig 4) Since all strains of *P aeruginosa* including rough variants and regardless of growth phase produce CA with no detectable differences in the St-Ag/St-Ab reference system it is probable that it is differences in the patients immune response which are responsible for the varying individual correlation between the antibody response against CA and other *P aeruginosa* antigens and between the antibody response against the common cross-

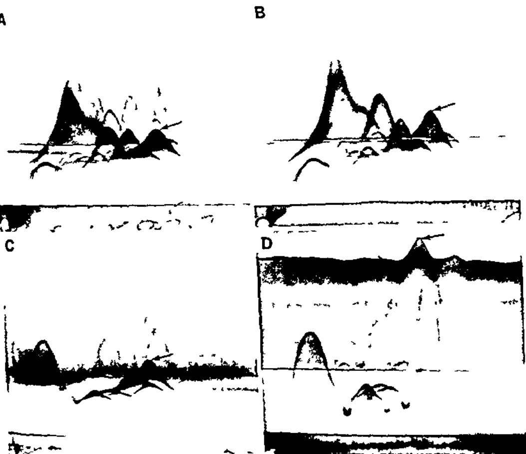


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C Same as *A* but with serum from another patient suffering from chronic *P aeruginosa* lung infection in the second dimension reference gel (10 µl/cm²) 17 precipitating antibodies were detectable in this serum including antibodies against CA (arrow) of the same titre as in *A* Saline in the intermediate gel

D Same as *C* but with antigens from *E coli* (20 µl/cm²) in the intermediate gel Compared with *C* the area of the CA precipitate (arrows) increased greatly after absorption of cross reactive antibodies by *E coli* antigens The area of a few other precipitates corresponding to other cross reactive *P aeruginosa* antigens (ref 3) have also increased

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	75–50%		5	4	3	
	50–25%		7	7	12	
	0%[b]		3	3	3	24

[a]) Number of *P aeruginosa* antibodies in the 24 sera (range) 1–46 Titres of antibodies against CA (range) 2–7

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DISCUSSION

Common antigen of *P aeruginosa* has recently been purified and characterized as a protein with mol wt 665,000–900,000 consisting of polypeptide subunits of mol wt 60,000 (11, 12) According to *Kayser* (9), it is situated in the inner part of the cell envelope Because of the occurrence of CA in a wide range of predominantly gram-negative bacterial species and the presence of precipitating antibodies *against* CA in 54% of normal human sera (and also in rabbit sera), the immunological and pathophysiological roles of this antigen during infection are of interest

The present study shows that the initial antibody response in 62% of the patients does not include increased titre of CA antibodies (Fig 1) However later in the infection the majority of the patients develop CA antibodies but even then 29% of the patients with an antibody response against other *P aeruginosa* antigens do not produce increased titre of CA antibodies (Fig 2) 14 out of the 31 patients who did not develop CA antibodies during the initial antibody response (Fig 1) had low titre (normal) CA antibodies before the infection and this was also the case in 18 of the 29 patients who did not develop CA antibodies during the highest measured antibody response (Fig 2) In these latter patients it could have been expected that the *P aeruginosa* infection would have provoked a secondary "booster" response of CA antibodies

The individual variation of the antibody response to CA is also reflected in the absorption *in situ* results obtained with antigens from *E coli P mirabilis* and *H influenzae* i e some of the patients produced predominantly antibodies against the common cross-reactive part of CA, whereas other patients produced predominantly antibodies against the *Pseudomonas*-specific part of CA (Table I Fig 4) Since all strains of *P aeruginosa* including rough variants and regardless of growth phase produce CA with no detectable differences in the St Ag/St Ab reference system it is probable that it is differences in the patients immune response which are responsible for the varying individual correlation between the antibody response against CA and other *P aeruginosa* antigens and between the antibody response against the common cross

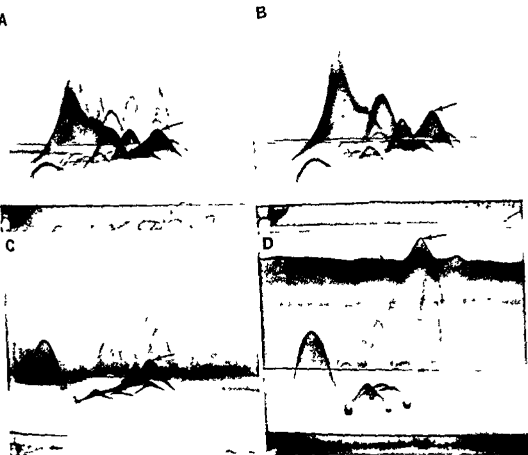


Fig 4 Absorption of antibodies *in situ* by means of crossed line immunoelectrophoresis

A Control plate showing 2 µl *P aeruginosa* antigens (St Ag) in the well run against serum from a patient suffering from chronic *P aeruginosa* lung infection in the second dimension reference gel (10 µl/cm²) 25 precipitating antibodies were demonstrable in the serum including antibodies against CA (arrow) Saline in the intermediate gel

B Same as *A* but with antigens from *E coli* (20 µl/cm²) in the intermediate gel Compared with *A* the area of the CA precipitate (arrow) increased only slightly after absorption of cross reactive antibodies by *E coli* antigens The area of a few other precipitates corresponding to other cross reactive *P aeruginosa* antigens (ref 3) have also increased

C Same as *A* but with serum from another patient suffering from chronic *P aeruginosa* lung infection in the second dimension reference gel (10 µl/cm²) 17 precipitating antibodies were detectable in this serum including antibodies against CA (arrow) of the same titre as in *A* Saline in the intermediate gel

D Same as *C* but with antigens from *E coli* (20 µl/cm²) in the intermediate gel Compared with *C* the area of the CA precipitate (arrows) increased greatly after absorption of cross reactive antibodies by *E coli* antigens The area of a few other precipitates corresponding to other cross reactive *P aeruginosa* antigens (ref 3) have also increased

Conclusion The first patient (A and B) produced predominantly antibodies against the *Pseudomonas* specific part of CA whereas the second patient (C and D) produced predominantly antibodies against the cross reactive part of CA

reactive part of the *Pseudomonas*-specific part of CA The reason for the varying individual antibody response is not known but genetic differences in the immune response and the immunological tolerance phenomenon may be considered The same explanations may by offered as regards the results obtained with sera from patients suffering from infections caused by bacterial species other than *Pseudomonas* containing CA

According to the results of this work, the measurement of CA antibodies alone is not as reliable a tool in the diagnosis of *P aeruginosa*

TABLE I *Absorption of* P aeruginosa *CA Antibodies in 24 Human Sera*[a] *by Antigens of Five Other Bacterial Species*

		Origin of antigens and number of sera				
		P pseudoalcaligenes	*E coli*	*P mirabilis*	*H influenzae*	*S aureus*
Percentage Absorption of CA antibodies	100 %	24	7	8	6	
	100–75 %		2	2		
	75–50 %		5	4	3	
	50–25 %		7	7	12	
	0 %[b]		3	3	3	24

a) Number of *P aeruginosa* antibodies in the 24 *sera (range)* 1–46 Titres of antibodies against CA *(range)* 2 7
b) < 25 % absorption is considered insignificant (3)

Table I and examples are given in Fig 4 In all cases antigens from *P pseudoalcaligenes* could absorb all precipitating antibodies against CA whereas *S aureus* antigens could not absorb antibodies at all However using antigens from *E coli P mirabilis* and *H influenzae* the results obtained with sera from different patients varied Some of the patients produced solely or predominantly antibodies against the cross reactive part of CA while others produced predominantly antibodies against the *Pseudomonas*-specific part of CA (Table I) The degree of absorption of antibodies by these three gram negative rods was generally similar in the individual sera There was no correlation between the degree of absorption of antibodies against CA and the titre of CA antibodies or the total number of precipitins in the sera

Development of Precipitating Antibodies against CA in Serum from Patients Suffering from other Bacterial Infections

None of the four patients with infections caused by gram positive cocci developed CA antibodies whereas three (33 %) of the nine patients with infections caused by gram negative bacteria developed CA antibodies (infections caused by *N meningitidis* and *E coli* titre 3 and by *P stutzeri* titre 5)

DISCUSSION

Common antigen of *P aeruginosa* has recently been purified and characterized as a protein with mol wt 665 000–900 000 consisting of polypeptide subunits of mol wt 60 000 (11 12) According to *Kayser* (9) it is situated in the inner part of the cell envelope Because of the occurrence of CA in a wide range of predominantly gram negative bacterial species and the presence of precipitating antibodies against CA in 54 % of normal human sera (and also in rabbit sera) the immunological and pathophysiological roles of this antigen during infection are of interest

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AN INDIRECT HAEMAGGLUTINATION TEST FOR DEMONSTRATION OF GONOCOCCAL ANTIBODIES USING GONOCOCCAL PILI AS ANTIGEN

II Serological Investigation of Patients Attending a Dermato-venereological Out-patients Clinic in Copenhagen

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Reimann K Lind I & Andersen K E An indirect haemagglutination test for demonstration of gonococcal antibodies using gonococcal pili as antigen II Serological investigation of patients attending a dermato-venereological out patients clinic in Copenhagen Acta path microbiol scand Sect. C *88* 155–162 1980

A total of 1223 serum specimens were obtained from 649 consecutive patients attending a dermato venereological out patient clinic in Copenhagen with a request for venereal disease control The sera were examined for gonococcal antibodies by both a gonococcal complement fixation test (GCF) and an indirect haemagglutination test using gonococcal pili as antigen (IHA) The diagnosis of current gonococcal infection in 28 per cent of the patients was based on positive culture for *Neisseria gonorrhoeae* from one or more of the following sites urethra, rectum and fauces in all patients and/or cervix in female patients The specificity sensitivity and predictive values of positive and negative test results were calculated for the various groups of patients on the assumption that all positive results both in patients without gonorrhoea but with a previous gonococcal infection and in patients without known current or previous infection, were false positives The following values were found *Sensitivity* IHA 45–100% GCF 6–29% *Specificity* IHA 65–89% GCF 97–100% *Predictive value of positive test result* IHA 43–74% GCF 69–100% *Predictive value of negative test result* IHA 78–100% GCF 61–85%

Key words Gonococcal antibodies indirect haemagglutination gonococcal pili

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During the period 1928–1978 a gonococcal complement fixation test (GCF) has been performed as routine test for the detection of gonococcal antibodies in our laboratory (17) About 1930 the sensitivity of GCF was found to be 44–56 per cent and 69–100 per cent when sera from male and female patients with uncomplicated or complicated gonorrhoea were examined In a recent study of similar groups of patients the sensitivity was only 2–5 and 21–30 per cent, the highest percentage being found for female patients with complicated gonococcal infection (pharyngeal gonorrh

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an indi… haemagglutination test using gonococcal pili as antigen (14 16), the present study was initiated Paired serum specimens from a »high risk group« of individuals were examined both by the GCF and the indirect haemagglutination (IHA)

infection as the detection of the total number of precipitating antibodies against *P aeruginosa* as currently used in our laboratory (7) This is because 29% of the *P aeruginosa* infections will not be diagnosed by measuring CA antibodies (false negative results) and possibly 33% of infections caused by other bacteria containing CA may be detected (false positive results) The results indicate that by using CA from *P aeruginosa* it would not be possible to detect serologically the majority of infections caused by bacteria containing this anti gen However, as it is easy to purify CA (11) it would seem of interest to investigate whether a pool of corresponding CA from common pathogenic bacterial species would improve the possibility of diagnosing infections caused by these species by measuring the antibody response to both the common cross reactive part and the species or genus specific part of CA

This investigation was supported by grants to D S from the *Danish Ministry of Education* and to J B H from the *Danish Medical Research Council* Mrs *Anni Bethlen* is thanked for skilful technical assistance

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12 *Sompolinsky D Hertz J B Høiby N Jensen K & Mansa B* An antigen common to a wide range of bacteria 2 A biochemical study of a common antigen from *Pseudomonas aeruginosa* In press

TABLE 1 *Distribution According to Age Sex Current and Previous Gonococcal Infection (gc) of 649 Consecutive Patients Attending a Dermato venereological Out patient Clinic in Copenhagen with a Request for Venereal Disease Control*

Patients	Females			Males		
	No	Age (years)		No	Age (years)	
		Mean	Range		Mean	Range
Without current gc	167	26 7	14–57	298	29 6	16–71
With current gc	86	25 1	15–57	98	28 5	16–53
Total	253	26 1	14–57	396	29 3	16–71

TABLE 2 *Distribution According to Reasons for Attending a Dermato-venereological Out-patient Clinic in Copenhagen with a Request for Venereal Disease Control (649 Consecutive Patients)*

	FEMALES		MALES	
	number of patients	number with current gc	number of patients	number with current gc
Symptoms of lower genital tract infection	35	8	138	51
Named contact	60	38[a)]	64	17[b)]
Concern about being disease carrier	54	6	48	8
Other reasons	104	34	146	22
Total	253	86	396	98

[a)] 63% of named female contacts
[b)] 27% of named male contacts

The diagnosis of current gc was made in 86/253 (34%) of female patients and 98/396 (25%) of male patients Two or more sets of specimens were obtained from 75% of the patients Ten patients seven males and three females had pharyngeal gonorrhoea From 81/86 (94%) of the female and 94/98 (96%) of the male patients gonococci were isolated from the first set of specimens The remaining nine patients were culture positive at the second examination the four males and two of the females being positive only from fauces and/or rectum The percentage of male patients with symptoms from the lower genital tract (35%) was, as expected, higher than the percentage of female patients with such symptoms (14%) Seventeen out of 64 (27%) named male contacts and 38 out of 60 (63%) named female contacts had current gc (Table 2)

Previous gonococcal infection was registered in 73/253 (29%) of the female and in 177/396 (45%) of the male patients No correlation could be registered between the antibody level measured (the titre) and the time elapsed since the last known gc Therefore, the only distinction made is plus or [illegible] citizens and consequently these results are not considered separately

Serological Tests

In the tables and figures only results obtained for one serum specimen per patient are registered If two or more serum specimens were obtained, as was the case for 75 per cent of the patients, the highest value among positive test results was used For nine patients with current gc, the sera obtained on the first occasion showed a negative reaction in

tests The results were evaluated for the various groups of patients by calculation of specificity, sensitivity and predictive values of positive and negative test results (19)

MATERIALS AND METHODS

Indirect haemag[illegible]

and *SS 35209/1975* type 2 colonies A mixture of these two pili preparations was used throughout this study *The performance of the test was as described previously* (16), with a few modifications a) Prior to use, the antigen was sonicated for 30 seconds at 0 °C using a MSE ultrasonic disintegrator (8 micron), b) The agglutination pattern was read after three hours The titre of a given serum specimen was recorded as the reciprocal value of the lowest serum concentration giving + reaction Titres ≧ 40 were considered positive

Gonococcal complement fixation test (GCF) The antigen was prepared and the test performed as described by *Reyn* (17) The highest serum concentration tested was 1 12 The titre was read as the reciprocal value of the lowest serum concentration giving 60 per cent haemolysis

Groups of patients studied During the period March 1976 – February 1977, all patients attending a dermato-venereological out-patients clinic in Copenhagen for one or more of the following reasons were included in the study a) symptoms of lower genital tract infection named contacts to patients with gonorrhoea c) concern about being disease carrier, d) symptoms of other sexually transmitted diseases with or without request for venereal disease control About 75 per cent of the patients returned for control examination(s) On each occasion blood samples were drawn and specimens were collected for bacteriological examination The diagnosis of current gonococcal infection was based on positive culture for *Neisseria gonorrhoeae* from one or more of the following sites urethra, rectum and fauces in all patients and/or cervix *in female patients Specimens for* bacteriological examination were taken on charcoal-impregnated swabs and transported in a modified Stuarts medium (17) The transportation time never exceeded 6 hours Each specimen was inoculated onto both selective and non-selective medium, and gonococcal like oxidase positive colonies were identified as gonococci by a direct immunofluorescence test (13) Furthermore suspected colonies from pharyngeal specimens were subjected to bacteriological identification (Gram stained smear carbohydrate utilization tests)

Statistics Calculations of specificity, sensitivity and predictive values of positive and negative test results were performed as described by *Vecchio* (19) These terms are defined as follows

$$\text{SPECIFICITY} = \frac{\text{Non-diseased persons negative to the test}}{\text{All non-diseased persons tested}} \times 100$$

$$\text{SENSITIVITY} = \frac{\text{Diseased persons with positive test}}{\text{All diseased persons tested}} \times 100$$

$$\text{PREDICTIVE VALUE OF POSITIVE TEST} = \frac{\text{Number of diseased persons with positive test}}{\text{Total number of persons with positive test}} \times 100$$

$$\text{PREDICTIVE VALUE OF NEGATIVE TEST} = \frac{\text{Number of non diseased persons with negative test}}{\text{Total number of persons with negative test}} \times 100$$

RESULTS

Study Population

Age and sex of the patients in relation to the prevalence of current and previous gonococcal infection (gc) are shown in Table 1, and Table 2 shows the distribution into groups according to the reasons for attending the clinic Three female and 32 male patients were repeaters, *i e* they attended the clinic on at least two separate occasions during the study period Only results obtained at one episode are included in the material, *i e* those obtained at the first visit or at the visit on which gonorrhoea was demonstrated

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Seventeen per cent of patients attending the clinic were foreigners The results obtained for this group did not differ from those obtained for Danish citizens and consequently these results are not considered separately

Serological Tests

In the tables and figures, only results obtained for one serum specimen per patient are registered If two or more serum specimens were obtained, as was the case for 75 per cent of the patients, the highest value among positive test results was used For nine patients with current gc, the sera obtained on the first occasion showed a negative reaction in

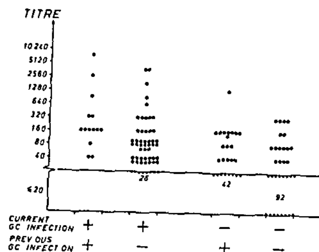


Fig 1 Results obtained by the indirect haemagglutination test for demonstration of gonococcal antibodies using gonococcal pili as antigen by examination of sera from 253 female patients attending a dermato venereological out patients clinic with a request for venereal disease control

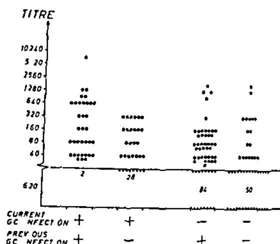


Figure 2 Results obtained by the indirect haemagglutination test for demonstration of gonococcal antibodies using gonococcal pili as antigen by examination of sera from 396 male patients attending a dermato venereological out patients clinic with a request for venereal disease control

IHA whereas a positive test result was obtained on the second occasion (1–7 weeks later)

Indirect haemagglutination test (IHA) Figure 1 shows the results obtained by examination of sera from 253 female patients Sera from all 14 patients with both current and previous gc gave positive results and of patients with current gc but without previous gc 46/72 (64%) were found to be positive Out of the 26 patients with current gc and negative serological test result 13 attended the clinic only once In the two groups of patients without current gonococcal infection a total of 33 positive test results were demonstrated Seventeen gave information about previous gc and eight of the remaining 16 were named contacts to patients with current gc The corresponding figures for 396 male patients are shown in Figure 2 About 50% of male patients with current gc did not return for control examination In the group of patients without current or previous gc sera from 18 patients gave positive results seven of these were named contacts It should be noted that sera from nine of the ten patients with pharyngeal gonorrhoea gave a positive reaction in the IHA

Gonococcal complement fixation test (GCF) Tables 3 and 4 show the number of positive results obtained by IHA and GCF for the various groups of patients All 26 sera which showed a positive GCF were positive by IHA No correlation could be registered between the level of antibodies detected by the two methods The percentage of GCF positive test results in the various groups of patients with current gc was low (6–29%) Only two sera out of ten from patients with pharyngeal gonorrhoea gave a positive result by the GCF test

Comparison of IHA and GCF Table 5 shows the

TABLE 3 *Comparison of Results Obtained by IHA (Indirect Haemagglutination Test Using Gonococcal Pili as Antigen) and GCF (Gonococcal Complement Fixation Test) 184 Serum Specimens from 184 Patients with Current Gonococcal Infection (gc)*

Sex	Previous gc	Number of patients	Number of positive reactions in IHA	GCF
F	+	14	14	4
	−	72	46	4
M	+	47	35	9
	−	51	23	4

TABLE 4 *Comparison of Results Obtained by IHA (Indirect Haemagglutination Test Using Gonococcal Pili as Antigen) and GCF (Gonococcal Complement Fixation Test) 465 Serum Specimens from 465 Patients without Current Gonococcal Infection (gc)*

Sex	Previous gc	Number of patients	Number of positive reactions in	
			IHA	GCF
F	+	59	17	1
	−	108	16	0
M	+	130	46	4
	−	168	18	0

sensitivity and the predictive values of positive test results for this material of »high risk« individuals The figures were calculated on the assumption that all positive test results obtained both for patients without current gc but with previous gc for patients without known current or previous gc were false positives The sensitivity of IHA was 45–100% and for GCF only 6–29% The predictive value of a positive IHA was 43–74% and that of a positive GCF 69–100% Table 6 gives the figures obtained by calculation of specificity and predictive values of negative test for the same groups of patients the predictive value of a negative IHA being 78–100%

DISCUSSION

The potential application of any serological test diagnosis casefinding or screening is a primary consideration in *its selection (5 7 9 20) In the* present context use for diagnosis means the establishment of the presence of suspected gonorrhoea in a patient with compatible symptoms or recent exposure to gonorrhoea Case detection is the testing of patients who have sought health care for some other purpose *e g* at antenatal or contraceptive clinics Screening involves the testing of volunteers from the general population In an analysis of characteristics required for an adequate screening test for gonorrhoea *Galen & Gambino* (7) state that the minimum requirements include a sensitivity greater than 80% a specificity greater than 99% and the ability to differentiate recent from old infection Serological tests using whole cells or cr[illegible]

[illegible]

in order to improve the specificity and sensitivity of serological tests for gonorrhoea current research in this field has concentrated on use of purified antigens primarily gonococcal pili The properties of gonococcal pili and their antigenic relationship have been reviewed by *Buchanan* (3) A high degree of heterogeneity was demonstrated when the antigenic structure of pili from different gonococcal strains was investigated using rabbit antibodies to purified pili *Buchanan* reported that shared antigens accounted for 2 5% or less (3) Despite this fact pili antigen from a single strain detected antibodies to gonococcal pili in 89% and 86% of asymptomatic female patients with gonorrhoea (4 15) In the present study pili antigen from two randomly selected strains was used and antibodies were demonstrated in similar high proportions of patients with gonorrhoea Close correlation was found between results obtained by the radioimmuno assay using pili antigen from one strain isolated in U S A (4) and the indirect haemagglutination (IHA) [illegible]

[illegible]

antigen and the complement fixation test using whole gonococcal cells as antigen (GCF) was made in the present study The sensitivity of the IHA [illegible]

[illegible]

females without previous gc and 29% in females with previous gc (Table 5) The specificity of the IHA varied between 65% in males with previous gc and 89% in males without previous gc The specificity of the GCF was approximately 100% for all four groups of patients (Table 6) The predictive values of positive and negative test results (19) were calculated on the [illegible]

[illegible] from a positive serological test result was unrelated to exposure to gonorrhoea was low since 8 out of 16 female and 7

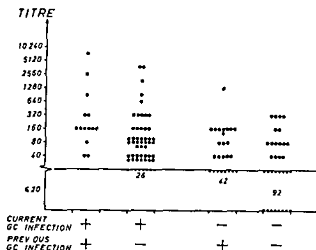


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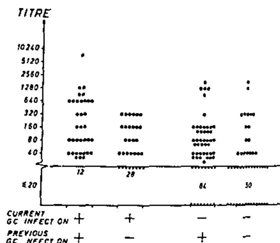


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	−	72	46	4
M	+	47	35	9
	−	51	23	4

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Sex	Previous gc	Number of patients	Number of positive reactions in IHA	GCF
F	+	59	17	1
	–	108	16	0
M	+	130	46	4
	–	168	18	0

ensitivity and the predictive values of positive test esults for this material of »high risk« individuals The figures were calculated on the assumption that all positive test results obtained both for patients without current gc but with previous gc for patients without known current or previous gc were false positives The sensitivity of IHA was 45–100% and for GCF only 6–29% The predictive value of a positive IHA was 43–74% and that of a positive GCF 69–100% Table 6 gives the figures obtained by calculation of specificity and predictive values of negative test for the same groups of patients the predictive value of a negative IHA being 78–100%

DISCUSSION

The potential application of any serological test diagnosis casefinding or screening is a primary consideration in its selection (5 7 9 20) In the present context use for diagnosis means the establishment of the presence of suspected gonorrhoea in a patient with compatible symptoms or recent exposure to gonorrhoea Case detection is the testing of patients who have sought health care for some other purpose e g at antenatal or contraceptive clinics Screening involves the testing of volunteers from the general population In an analysis of characteristics required for an adequate screening test for gonorrhoea *Galen & Gambino* (7) state that the minimum requirements include a sensitivity greater than 80% a specificity greater than 99% and the ability to differentiate recent from old infection Serological tests using whole cells or crude extracts of *N gonorrhoeae* as antigens do not fulfil any of these conditions as discussed recently by a WHO Scientific Group (20)

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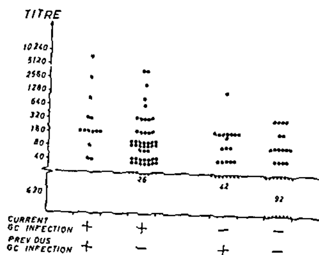


Fig 1 Results obtained by the indirect haemagglutination test for demonstration of gonococcal antibodies using gonococcal pili as antigen by examination of sera from 253 female patients attending a dermato venereological out patients clinic with a request for venereal disease control

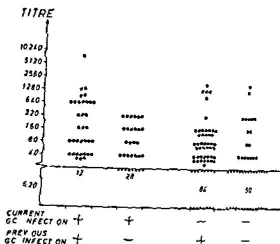


Figure 2 Results obtained by the indirect haemagglutination test for demonstration of gonococcal antibodies using gonococcal pili as antigen by examination of sera from 396 male patients attending a dermato venereological out patients clinic with a request for venereal disease control

IHA whereas a positive test result was obtained on the second occasion (1–7 weeks later)

Indirect haemagglutination test (IHA) Figure 1 shows the results obtained by examination of sera from 253 female patients Sera from all 14 patients with both current and previous gc gave positive results and of patients with current gc but without previous gc 46/72 (64%) were found to be positive Out of the 26 patients with current gc and negative serological test result 13 attended the clinic only once In the two groups of patients without current gonococcal infection a total of 33 positive test results were demonstrated Seventeen gave information about previous gc and eight of the remaining 16 were named contacts to patients with current gc The corresponding figures for 396 male patients are shown in Figure 2 About 50% of male patients with current gc did not return for control examination In the group of patients without current or previous gc sera from 18 patients gave positive results seven of these were named contacts It should be noted that sera from nine of the ten patients with pharyngeal gonorrhoea gave a positive reaction in the IHA

Gonococcal complement fixation test (GCF) Tables 3 and 4 show the number of positive results obtained by IHA and GCF for the various groups of patients All 26 sera which showed a positive GCF were positive by IHA No correlation could be registered between the level of antibodies detected by the two methods The percentage of GCF positive test results in the various groups of patients with current gc was low (6–29%) Only two sera out of ten from patients with pharyngeal gonorrhoea gave a positive result by the GCF test

Comparison of IHA and GCF Table 5 shows the

TABLE 3 *Comparison of Results Obtained by IHA (Indirect Haemagglutination Test Using Gonococcal Pili as Antigen) and GCF (Gonococcal Complement Fixation Test) 184 Serum Specimens from 184 Patients with Current Gonococcal Infection (gc)*

Sex	Previous gc	Number of patients	Number of positive reactions in IHA	GCF
F	+	14	14	4
	−	72	46	4
M	+	47	35	9
	−	51	23	4

TABLE 4 *Comparison of Results Obtained by IHA (Indirect Haemagglutination Test Using Gonococcal Pili as Antigen) and GCF (Gonococcal Complement Fixation Test) 465 Serum Specimens from 465 Patients without Current Gonococcal Infection (gc)*

Sex	Previous gc	Number of patients	Number of positive reactions in IHA	Number of positive reactions in GCF
F	+	59	17	1
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In order to improve the specificity and sensitivity of serological tests for *gonorrhoea current* research in this field has concentrated on use of purified antigens primarily gonococcal pili The properties of gonococcal pili and their antigenic relationship have been reviewed by *Buchanan* (3) A high degree of heterogeneity was demonstrated when the antigenic structure of pili from different gonococcal strains was investigated using rabbit antibodies to purified pili *Buchanan* reported that shared antigens accounted for 25% or less (3) Despite this fact pili antigen from a single strain detected antibodies to gonococcal pili in 89% and 86% of asymptomatic female patients with gonorrhoea (4 15) In the present study pili antigen from two randomly selected strains was used and antibodies were demonstrated in similar high proportions of patients with gonorrhoea Close correlation was found between results obtained by *the radioimmuno* assay using pili antigen from ...

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TABLE 5 *Serological Screening of 253 Female and 396 Male Patients with or without Previous Gonococcal Infection (gc)*

Sex	Previous gc	IHA		GCF	
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M	+	74	43	19	69
	−	45	56	8	100

IHA indirect haemagglutination test
GCF gonococcal complement fixation test

out of 18 male patients without current or previous gc were named contacts to patients with gonorrhoea

Using the quantitative radioimmuno assay for determination of antibody to gonococcal pili (4) in sera from a low risk population of female patients, *Holmes et al* (9) found a sensitivity of 65% and a predictive value of positive test result of 14% They conclude that the major current limitations for gonorrhoea case detection by examination of a single serum specimen appear to be the persistent antibodies from past infection and antibodies cross-reacting with *N meningitidis* The positive test results occurred with increased frequency in meningococcal carriers However, to the knowledge of the authors, shared antigenicity of gonococcal and meningococcal pili has not yet been demonstrated

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A serological test for gonorrhoea with the ability to differentiate between current and previous gonococcal infection does not seem to be attainable Antibodies to gonococcal pili persist for years as demonstrated in the present study and by others (9) Thus, in this respect no profit was derived from the

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	−	89	84	100	78

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In conclusion serological tests using gonococcal pili antigen seem to be superior with regard to specificity Due to the short incubation period of gonorrhoea and the persistance of antibodies for years a serological test cannot be used as the only basis for diagnosis of acute cases or for screening purposes Major potential fields for application of the IHA are the diagnosis of complicated gonococcal infections case finding in population groups with a prevalence of gonorrhoea of more than 2 per cent and sero-epidemiological studies in developing countries (5 9 20)

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Using the quantitative radioimmuno assay for determination of antibody to gonococcal pili (4) in sera from a low risk population of female patients, *Holmes et al* (9) found a sensitivity of 65% and a predictive value of positive test result of 14% They conclude that the major current limitations for gonorrhoea case detection by examination of a single serum specimen appear to be the persistent antibodies from past infection and antibodies cross-reacting with *N meningitidis* The positive test results occurred with increased frequency in meningococcal carriers However, to the knowledge of the authors, shared antigenicity of gonococcal and meningococcal pili has not yet been demonstrated

Gonorrhoea has a short incubation period resulting in a time interval between onset of disease and the presence of circulating antibodies which influence the sensitivity of a serological test In the present study this is probably the explanation for some of the negative serological results for patients with current gonococcal infection (Fig 1 and 2) since 50% of these patients did not return for control examination This means that they were tested only once and often within one week after exposure to gonorrhoea

A serological test for gonorrhoea with the ability to differentiate between current and previous gonococcal infection does not seem to be attainable Antibodies to gonococcal pili persist for years as demonstrated in the present study and by others (9) Thus, in this respect no profit was derived from the

TABLE 6 *Serological Screening of 253 Female and 396 Male Patients with or without Previous Gonococcal Infection (gc)*

Sex	Previous gc	IHA		GCF	
		Specificity %	Predictive value of negative test %	Specificity %	Predictive value of negative test %
F	+	71	100	98	85
	−	85	78	100	61
M	+	65	88	97	77
	−	89	84	100	78

IHA indirect haemagglutination test
GCF gonococcal complement fixation test

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LYMPHOCYTE SUBPOPULATIONS IN MAN CHARACTERIZATION OF *IN VIVO*-EDUCATED, ALLOREACTIVE, CYTOTOXIC LYMPHOCYTES

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Johnsen H E & Madsen M Lymphocyte subpopulations in man Characterization of *in v vo* educated alloreactive cytotoxic lymphocytes Acta path microbiol scand Sect. C *88* 163–171 1980

Alloreactive cytotoxic lymphocytes present in peripheral blood of two normal humans have been stud ed by rosette fractionation experiments It is shown that the effector cells have receptors for SRBC low av d ty FcR are nylon non adherent and w ho CR There
membrane m
the effector
immune syst
specif c others non specific in paral el to I helper and suppressor lymphocytes

Key words Lymphocyte subpopulations characterization

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Accepted as subm tted 23 i 80

Several lymphocyte mediated cytolytic phenomena have been described The cytolysis is a result of an intimate contact between effector cells and a relevant target cell through an effector cell receptor with affinity for target membrane structures followed by an effector cell dependent irreversible damage of the target cell membrane (4)

The source of the target cell as well as the attached membrane structures has given a natural d vision of the phenomena into (a) *antibody dependent cell mediated cytotoxicity* (ADCC) against antibody sensitized allo- and xenogeneic target cells where the receptor of the effector cells specific for the Fc part of target bound IgG is the Fc receptor (FcR) (21) (b) *spontaneous or natural cell mediated cytotoxicity* (SCMC) where the target cells have been long time cultured allo- or xenogeneic tumor cell lines or fibroblasts The receptors and target determinants responsible for this phenomenon have not been characterized (11 29 30 33) (c) *mitogen induced cell med ated cytotoxicity* (MICC) is possibly mediated through binding of a bi or polyvalent mitogen to both the effector and the allo- or xenogeneic target cell Alternatively the mechanism behind this phenomenon could be a mitogen dependent (activation) restoration of the cytotox c potential of effector cells with low or no detectable natural cytotoxicity (7 9 35) (d) *immune T lymphocyte mediated cytotoxicity* (LMC) via binding of the immune T cell receptor to membrane determinants on relevant target cells The target cells have most often been blast transformed lymphocytes and the target structures bound to the T cell receptor have been characterized as membrane structures coded from the major histocompatibility complex (MHC) (3 10)

Characterization of the different cytotoxic effector cells has been performed with the help of surface membrane markers and antigens Although the

surface structures of *Neisseria gonorrhoeae* In Immunobiology of *Neisseria gonorrhoeae* ASM Washington DC 1978 p 93–100
19 *Vecchio T J* Predictive value of a single diagnostic test in unselected populations New Engl J Med *274* 1171–1173 1966
20 *WHO Scientific Group* *Neisseria gonorrhoeae* and gonococcal infections Technical Report Series 616 World Health Organization Geneva 1978

Experimental procedure for the cytotoxicity tests The fundamental technical set up for the cytotoxicity tests is as described previously (16)

Preparation of target cells 3 × 10⁶ lymphocytes in 2 ml RPMI 1640 (Gibco Bio-Cult) supplemented with penicillin 100 IU/ml streptomycin 100 µg/ml glutamine 2 mM and 25% (v/v) pooled heat inactivated human serum (SRPMI) were stimulated with phytohaemagglutinin (Wellcome UK in a final dilution 1 100 (v/v)) After 3 days in culture the lymphoblasts from several tubes were pooled washed twice and stored frozen in liquid nitrogen at a concentration of 3 × 10⁶ cells/ml as described by *Jørgensen & Lamm* (17) This procedure gave an accessible standardized target source After being thawed 1–2 × 10⁶ blasts were resuspended in the volume of $Na_2\,{}^{51}CrO_4$ containing 50 µCi (specific activity 100–350 mCi/mg Cr) incubated at 37 °C in a water bath for 30 min with frequent shaking followed by washing twice in TC-199 (buffered with bicarbonate and Hepes 10 mM of each and containing 2 mM glutamine 100 IU/ml penicillin and 100 µg/ml streptomycin pH 7 4) For ADCC the antibody sensitization of targets was performed by incubating at 37 °C for 30 min 10⁶ ⁵¹Cr marked blasts in 1 ml antiserum (see below) diluted 1 4 in TC 199 After being washed twice in TC 199 (500 *g* 10 min) the ⁵¹Cr marked antibody sensitized lymphoblast targets were resuspended in SRPMI and adjusted to 10⁵ cells/ml

Preparation of effector cells The cells to be tested for LMC and ADCC effector capacity – that is unfractiona ted enriched or depleted cell suspensions – were resuspended in SRPMI and adjusted to 5 × 10⁶ cells/ml if not otherwise stated

Exper mental procedure for LMC and ADCC 100 µl of the target cell suspension (~ 10⁴ cells) was mixed in duplicate plastic tubes (70/10 mm round bottomed catalogue No 1090 Nunc Roskilde Denmark) with 100 µl of the effector cell suspension (50 × 10⁴ cells) and centrifuged at 50 *g* for 5 min followed by incubation for 4 h in humidified 5% CO_2/95% atmospheric air at 37 °C

After incubation the effector target contact was disrupted and the experiments were stopped by adding 1 5 ml of cold (4 °C) 0 9% saline to each tube followed by storage overnight at 4 °C for convenience The spontaneous ⁵¹Cr release was only partly ascribed to this storage After centrifugation (1000 *g* 10 min) a 1 0 ml aliquot of each supernatant was transferred to a new tube leaving 0 7 ml plus the cell pellet in the test tubes The radioactivity in all tubes was counted in a Packard Auto-gamma-counter preset for counting either a total of 10⁴ counts or for 10 min

The maximal chromium release was obtained by freeze-thawing three times 10⁴ targets in 200 µl SRPMI After addition of 1 5 ml saline these tubes were treated like the test tubes The spontaneous release of ⁵¹Cr activity was evaluated from tubes with 10⁴ targets in 200 µl SRPMI without effector cells These tubes and the test tubes were evaluated in parallel

Calculation of percentage of cytotoxicity The percentage of ⁵¹Cr release in the tubes was calculated from the formula

⁵¹Cr release =

$$\frac{\text{activity released in the supernatant}}{\text{total activity in the tube}} \times 100\%$$

Since percentage ⁵¹Cr released from the test tubes = T percentage ⁵¹Cr released spontaneously = S and maximum percentage ⁵¹Cr released by the freeze thaw procedure = M then the percentage of cytotoxicity was calculated from the formula

$$\%\ \text{cytotoxicity} = T\ S$$

and maximum cytotoxicity which is the working range of the test was calculated from the formula

$$\%\ \text{maximum cytotoxicity} = M\ S$$

Antiserum One serum containing lymphocytotoxic antibodies as revealed by the lymphocytotoxic microtech nique were used The serum (No 26673/68) was multispecific and freeze-dried reconstituted by sterile water to the original volume and inactivated (56 °C 30 min) before use

as
A
11

(♀) and S P (♂) None of the donors have a family history of immunological disorders or genetic diseases H P is a 35 year-old female with a history of one pregnancy (1975) and no blood transfusions After delivery she developed a weak lymphocytotoxic anti HLA A2 antibody (titre 2–4) which disappeared approx 18 months after delivery and has not been present since In parallel H P developed lymphocytotoxic lymphocy tes with a specificity for target determinants associated to HLA A2 These cells were observed for the first time approx 2 years after pregnancy and were demonstrable during the whole period of this study although they previously have been absent in intervals of up to one month

S P is a 40 year-old male born in India on arrival in Denmark he was 20 years old He has no knowledge of receiving blood transfusions His cytotoxic cells have been demonstrated in long periods and absent in intervals up to three months During the period of this study cytotoxic cells were abolished in two experiments but were demonstrable each time one to two weeks later This male has been immunized once a year during the last fourteen years with blood group B substance (antigens) to produce an immune anti B serum used for blood grouping

effector cells operationally have been named killer cells (K cells) in ADDC natural killer cells (NK cells) in SCMC and cytotoxic T lymphocytes (CTL) in LMC the phenotypic characterization of these lymphocytes by the membrane markers has been controversial Trying to summarize recent studies in man (2 11 16 23 25 26 29 30 34, 35) it may be concluded that human K and NK-cells for the major part have receptors for sheep red blood cells (SRBC) and helix pomatia haemagglutinin although with the methods available effector cells have been identified without these receptors There seems to be no doubt that most of the K and NK-cells have FcRs of high avidity but are without receptors for complement (CR) they are non adherent to nylon wool and without easily detectable surface membrane immunoglobulin (SmIg) The effector cells in MICC seem to have the same characteristics although the CR and SmIg have not been studied (9 12 35) Little is known about the effector cell in LMC in man although by definition it has to be a T cell Recent studies in mice have shown that a part of the cytotoxic T cells have FcRs or generate FcRs both during *in vivo* and *in vitro* generation (18 19 28 32) This marker on CTLs raises the question whether the receptor molecule responsible for cytotoxicity is a cytophilic IgG molecule bound through the FcR Studies of the alloreactive cytotoxic effector cells in man have mostly been performed with *in vitro* educated cytotoxic lymphocytes again showing discrepancies Some reports (5) describe a significant influence of a FcR positive T cell for the generation of CTLs although the CTL do not necessarily present the FcR others describe no influence of FcR positive responding T cells for generation of CTLs (31 own unpublished observation)

By help of membrane markers and fractionation experiments the object of this study is to characterize human *in vivo* generated CTLs with alloreactivity

MATERIALS AND METHODS

Isolation of monocuclear cells Cells were isolated as described by *Boyum* with local modifications as described previously (13 17) Briefly defibrinated blood was diluted with an equal volume of balanced salt solution with Tris (BSS) and placed on Lymphoprep® (Nyegaard & Co A/S Oslo) After 20 min of centrifugation at 1000 *g* mononuclear cells were harvested from the interphase washed twice in BSS (500 *g* 10 min) and resuspended to 3 × 10⁶ cells/ml in BSS unless otherwise stated

Quantitation of E EA EAC rosette forming cells (RFC) The rosette tests were performed as described previously (14 22) The indicator cells for the E rosette test were 2 aminoethylisothiuronium bromide (AET)-treated sheep erythrocytes (SRBC) for the EA rosette test ox erythrocytes (ORBC) sensitized with subagglutinating amounts of a hyperimmune rabbit anti-ORBC antiserum containing antibodies of the IgG class were used and for the EAC rosette test ORBC were sensitized with zymosan treated normal human serum (RJ) which contains antibodies of the IgM class and is non haemolytic owing to lack of C5 The indicator cells were resuspended to 1% (v/v) in BSS with fetal calf serum (FCS) E in 40% FCS EA and EAC in 10% FSC

The rosette tests were performed in duplicate tubes by mixing 100 µl of the lymphocyte suspension and 100 µl of the indicator cell suspension which was centrifuged at 50 *g* for 5 min and incubated overnight at 4 °C Enumeration of RFC was done after toluidine blue staining and resuspension counting a total of 200 lymphocytes

Quantitation of SmIg positive lymphocytes SmIg positive lymphocytes were quantitated by the direct immunofluorescence technique of *Papamichail et al* (24) which was slightly modified Briefly 1–2 × 10⁶ lymphocytes were incubated at 4 °C for 30 min in 100 µl fluorescein isothiocyanate (FITC)-conjugated polyvalent rabbit anti human Ig (Dakopatts Denmark) diluted 1 10 After two washes SmIg positive lymphocytes were scored in wet preparations counting 200 lymphocytes in a fluorescence microscope

Depletion of adherent cells by nylon wool column fraction A 10 ml plastic syringe was packed with 0 5 g nylon wool (Fenwal Laboratories) and two times 10 ml BSS was passed through the nylon by the syringe piston packing the wool to 3 ml After preincubation at 37 °C the column was ready for use About 30 × 10⁶ cells in 3 ml BSS with 10% FCS were prewarmed at 37 °C and applied on the nylon wool column After 45 min of incubation at 37 °C the non adherent cells were passively eluted by twice adding 3 ml BSS with 10% FCS (prewarmed to 37 °C) After elution the non adherent cells were washed twice

[illegible] RFC 3 ml lymphocyte [illegible] 1 ml of [illegible] rifuga[illegible]tion at 200 *g* for 5 min incubation at 4 [illegible] 0 min and gentle resuspension the cells were flotated at 1000 *g* on Lymphoprep The non RFC were harvested at the interphase and washed twice The RFC were in the pellet and the E and EA indicaor cells were lysed by addition of fresh autologous serum (harvested from the initial isolation of mononuclear cells) and incubation at 37 °C for 15 min The EAC indicators could not be lysed by immune haemolysis and hypotonic lysis was performed by adding sterile water for 15–30 s followed by an equal volume of double osmotic phosphate buffered saline After haemolysis the remaining lymphocytes were washed twice

Trypsinization of effector cells 6 × 10⁶ lymphocytes in 2 ml BSS were mixed with 1 ml 0 5% (v/v) trypsin (Difco Laboratories Detroit MI) solution in BSS The cells were incubated at 37 °C for 45 min followed by washes in BSS

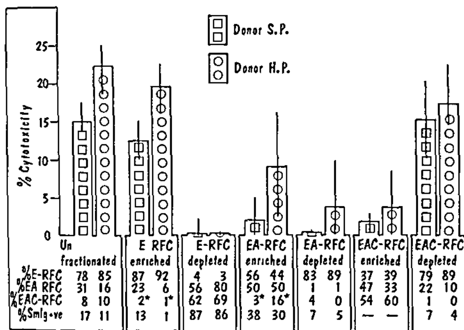


Fig 1 The cytotoxicity of effector cell suspensions from donor S P and H P before and after enrichment and depletion for E EA and EAC RFC The results are mean and range percentage cytotoxicity against target B and D for S P and A B and D for H P Maximum cytotoxicity was 48 8% (range 42 7–55 3%) The relative distribution of lymphocyte subpopulations in the different effector suspensions are given (★) indicate underestimation due to the immune haemolysis procedure during fractionation (–) not done

depleted suspensions thus the cytotoxic cells have receptor for SRBC but no CR which is in accordance with the fact that these two markers as they are used here are non-overlapping (15)

Further Study of the FcR Positive and Negative E-RFC

Both the EA RFC enriched and the depleted cell suspensions have a strongly reduced cytotoxicity In an attempt to explain this paradox dose response studies were performed with fractionated suspensions from one of the donors (H P) It can be seen in Fig 2 that the E RFC enriched suspension has an enhanced cytotoxic capacity compared to unfractionated cell suspensions while the E RFC depleted suspension has no cytotoxicity The E RFC enriched suspension was fractionated into EA RFC enriched and depleted effector cells giving results (Fig 2) in accordance with Fig 1 Both fractions have a low but nearly equal activity The explanation could be that the major cytotoxicity was in the EA RFC enriched suspension but the indicator cell ghosts (EA) around the effector cells inhibit the close contact necessary for lysis to occur Incubation overnight at 37 °C of the cell suspensions to elute

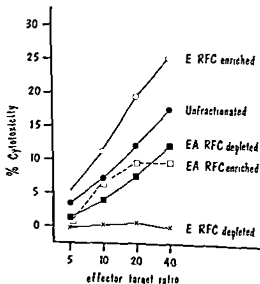


Fig 2 Dose response curves for LMC activity of rosette enriched and depleted cell suspensions The EA fractionation was performed on the E RFC enriched suspension The results are from one experiment with H P as effector against target B and D given as mean % values against the two targets

RESULTS

Two normal healthy donors, who by *experience* were known to have cytotoxic lymphocytes in peripheral blood against allogeneic target cells, have been tested in this study One of the donors (H P) has been described previously (20) HLA-D typing of her lymphocytes by homozygous typing cells was impossible owing to false assignments, as her cytotoxic cells actually killed the stimulator cells The target determinants identified by these cytotoxic cells were associated with HLA-A2 The same HLA-D typing problem was observed with another donor (S P) who also had cytotoxic cells in peripheral blood, the specificity of the cytotoxic cells is not known

The Cytotoxic Cells are Nylon Wool Non-adherent and Trypsin Sensitive

Testing these donors against a highly selected panel of PHA blast targets (Table 1) reveals »specificity«, as donor H P is positive against targets A, B and D, while donor S P was positive against B and D None of the donors are positive against autologous target cells Furthermore it can be seen from Table 1 that the major part of cytotoxic cells are nylon wool non adherent Finally, the cytotoxicity is abolished after trypsin treatment, indicating that the target binding structures (receptors) are trypsin sensitive

Characterization of the Effector Cells by Rosette Fractionation Experiments

Three rosette tests were used for gradient fractionation of the effector cell suspensions giving the results shown in Fig 1 It can be seen that the cytotoxic capacity is retained in the E RFC enriched and EAC-RFC depleted cell suspensions while it disappears or is reduced in the E- and EA RFC depleted and EA- and EAC-enriched suspensions

One important thing to have in mind before conclusions can be drawn from these results, is the influence of the fractionation procedure *per se* on the cytotoxic capacity of enriched or depleted cell suspensions The depleted suspensions are found with less than 4% rosette forming cells revealed by rerosetting, and the cells seem to be morphologically unchanged The cells in the E RFC enriched suspensions also seem to be rather unaffected by the fractionation procedure, with a purity of 85–95% whereas the EA- and EAC RFC enriched suspensions have a »purity« of 50–60% revealed by rerosetting Morphologically we could identify EA and EAC indicator cell ghosts fixed to the lymphocytes after the haemolytic procedure indicating that the FcR and CR are occupied by immune complexes and making these two suspensions useless for absolute conclusions concerning their effector capacities and purity

It can be concluded that nearly total cytotoxicity is retained in the E RFC enriched and EAC RFC

TABLE 1 *Cytotoxicity of Mononuclear Cells after Depletion of Nylon Wool Adherent Cells and Trypsin Treatment*

			Targets[a]			
			A	B	C	D
Effectors	Untreated cells	H P	14 7[b]	15 8	3 2	11 7
		S P	0 5	14 4	−2 9	16 8
	Nylon wool non adherent cells	H P	20 3	21 9	5 3	16 1
		S P	0 8	14 0	0 4	16 4
	Trypsin treated cells	H P	−0 9	4 2	−1 6	0 2
		S P	n d	n d	0 5	−2 0
	Maximum/spontaneous ^{51}Cr release		88 7/33 3	88 1/30 1	84 9/33 3	86 0/30 8

a) The HLA phenotype of the panel donors were
A HLA A2 w19 B5 40
B HLA A1 2 B8 13
C HLA A3 11, B7 35
D HLA A2 9 B12 15

b) The results are given as % cytotoxicity with the effector target ratio 30 1

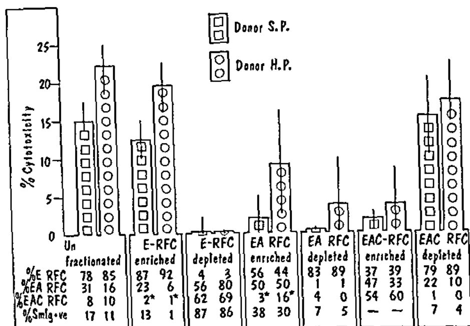


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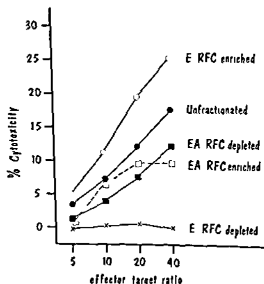


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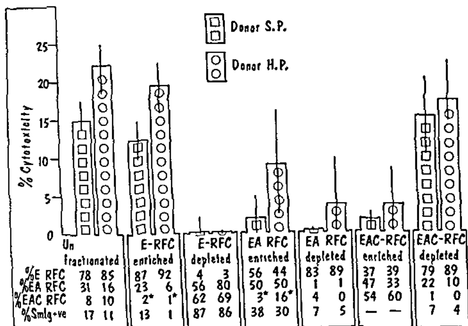


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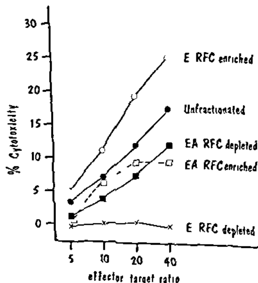


Fig 2 Dose response curves fo
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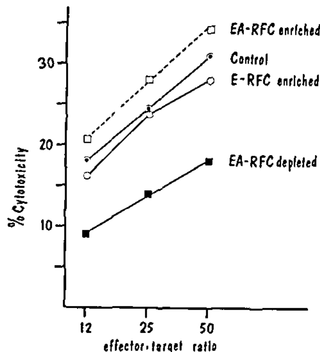


Fig 3 Dose response studies for LMC activity of the fractionated cell suspensions used in Fig 2, after 24 hours incubation at 37 °C Control suspension is the E RFC enriched suspension treated with the EA indicator cells without EA rosette fractionation

the indicator complexes from the effector cells followed by testing for cytotoxicity, gave the results shown in Fig 3 The EA-RFC enriched E RFC have more than four times the cytotoxic capacity of the non EA-RFC- In conclusion the major part of the cytotoxic cells have recognizable FcR

As trypsin treatment can abolish the cytotoxicity and the effector cells have FcR it may be reasonable, as previously mentioned, to ask whether the receptor can be a native IgG molecule unspecifically bound to the cells via the FcR If this is the explanation, then incubation of donor cells from individuals having no cytotoxic lymphocytes with serum from H P and S P should give the ocurrence of cytotoxic cells through a mechanism parallel to ADCC The results from such experiments are shown in Table 2, indicating that this was not the case This is further supported by the fact that non E-RFC (which have K cell activity (16)) from H P and S P do not act as CTLs (Fig 1 and 2)

In conclusion, if the receptor is an IgG molecule it may be bound specifically to a subpopulation of FcR positive cells alternatively the T cell receptors for the target cell determinants are trypsin sensitive and have no direct relation to the FcR found on the major part of the effector cells

The EA Binding Avidity of the FcR on the Cytotoxic Cells

Depletion of EA-RFC with EA indicator cells sensitized with varying dilutions of antisera will deplete the FcR positive cells dependent on the avidity of their FcR (16) Such depletion experiments were performed with results as shown in Fig 4 Depletion of cells binding EA sensitized with higher antiserum dilution than 1 320 (EA₃₂₀) enhanced the cytotoxicity against unsensitized target cells while it strongly abolished the ADCC capacity of the cell suspensions This is due to depletion of high avidity FcR positive cells which are mainly K cells (16) and a parallel concentration of the cytotoxic effector cells A further depletion with

TABLE 2 *LMC after Preincubation of Mononuclear Cells in Serum from Donor H P and S P*

Effector cell donors	Target panel				
	A	B	C	D	Dsens[a]
H P	22 6[b]	21 7	4 3	23 9	33 3
H P + Se(H P)[c]	22 6	22 0	7 0	21 9	37 1
N 1[d]	1 9	3 9	0 5	1 7	29 7
N 1 + Se(H P)	2 2	3 7	1 6	2 3	16 0
S P	4 8	25 1	5 2	23 5	21 2
S P + Se(S P)	5 7	33 2	6 2	19 8	20 7
N 2	1 1	3 1	0 0	2 6	27 4
N 2 + Se(S P)	1 7	3 1	0 6	3 3	20 9

a) Target D sensitized with HLA antisera
b) The results are given as % cytotoxicity
c) Mononuclear effector cells which have been incubated for 1 h at 37 °C in serum from donor H P
d) N is normal non cytotoxic donors only results from one representative normal donor as effectors is shown

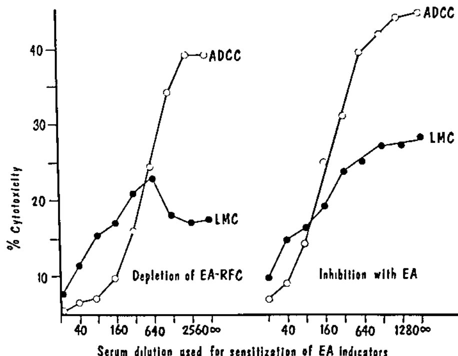


Fig 4 Correlation between the LMC and ADCC with cell suspensions depleted for EA RFC or inhibited with EA indicator cells The depletion or inhibition is performed with EA indicator cells sensitized with the anti-ox erythrocyte antiserum dilutions given ∞ indicates unsensitized erythrocytes The results are given as mean percentage cytotoxicity of donor H P against two targets (A and D) in LMC and one target (C sensitized with antibodies) in ADCC

EA_{320}-EA_{20} shows a paralllel depletion of effector cells acting both in ADCC and in LMC, indicating that these effector cells are either identical or two effector cell populations with identical FcR avidity These results were confirmed by blocking experiments where the effectors in ADCC and LMC were blocked after rosetting with varying sensitized EA indicator cells without fractionation (Fig 4)

DISCUSSION

This study of membrane markers on peripheral blood lymphocytes with cytotoxicity against allogeneic lymphoblasts shows that the effector cells in two persons have receptors for SRBC and low avidity FcR the cells are nylon wool non adherent and without CR Thus it is reasonable to conclude that these cells are cytotoxic T cells (CTLs) in accordance with the classification of *in vivo* educated cytotoxic lymphocytes with allospecificity in other species (3)

We have shown that th

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and CTLs on the cellular basis have not been fully explained in the present study However, it may be reasonable to assume that the FcR positive CTLs

cells with identical membrane marker phenotypes

It should be mentioned that in many experiments we found a minor residual cytotoxic capacity among EA RFC depleted T cells, reflecting either a real FcR negative subpopulation or an insensitive method for identification of the FcR of very low avidity The last possibility is suggested from Fig 4 as the cytotoxicity after depletion and inhibition of CTLs with EA indicator cells of varying sensitization does not end in a plateau, thus a further

human CTLs seems to be in accordance with results in mice, where a fraction of the

spleen T

and a gre

CTLs with allospecificity (18, 32) Furthermore, mixed lymphocyte culture alloactivated human T

lymphocytes have been described with ADCC (6) SCMC (27) and MICC (7) capacity

The FcR positive lymphocyte population is heterogeneous as the FcR is found both on cells with receptors for SRBC and SmIg and on cells without these membrane markers (15) although the occurrence of SmIg positive cells with FcR has been disputed (8) What seems interesting is that FcR positive non B lymphocytes include nearly all the cytotoxic effector lymphocytes known i e K NK CTLs and the effector cells in MICC The phenotypic relationship between these apparent *functionally different cell populations* indicates that they have some common ancestor in the immune system Lymphocyte-dependent cell mediated cyto toxicity is by definition a function of the thymus dependent limb of the efferent immune system and it may be logical to postulate that the above mentioned effector cells are T lymphocytes of origin and function Some cytotoxic lymphocytes then are specific others non specific in parallel to T helper and T suppressor cells

The partial lack of known established T cell markers i e SRBC receptors and T cell antigens on a minor part of K and NK cells in peripheral blood may only reflect insensitive methods for detection of cells with a very low number of surface membrane markers alternatively these cells may be pre or post thymic circulating T cells with hidden or still not present membrane markers of the T lineage Indeed neuraminidase treatment of lymphocytes reveals a higher number of E RFC compared to untreated lymphocytes (own unpublished observa tion) and PHA treatment of receptor negative cells can induce the receptor on these cells (1) These results seem to indicate that a functional view as the primary background for division of the lymphocy tes into two complementary but not independent compartments may give some clarification in the study of lymphocyte membrane marker pheno types

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CELLULAR IMMUNE RESPONSE IN RABBITS IMMUNIZED WITH PURIFIED VISNA VIRUS

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Thormar H Kristensson K Lin F H & Wisniewski H M Cellular immune response in rabbits immunized with purified visna virus Acta path microbiol scand Sect. C 88 173–177 1980

Visna virus sensitized lymphocytes were demonstrated in the peripheral blood of rabbits following immunization with purified concentrated visna virus Lymphocyte sensitization was maximal at about 2 weeks and fell to control levels in 5 weeks A delayed type cutaneous hypersensitivity to the virus was observed at 2 weeks and lasted for at least 9 weeks Both reactions increased after a booster injection Although sera from immunized rabbits had precipitating antibodies against visna virus protein P25 their lymphocytes were not stimulated by P25 However there was a slight reaction to P25 in the skin test Therefore in contrast to the humoral immune response P25 is apparently not the most active viral antigen in the CMI response in rabbits

Key words Visna virus cellular immune response lymphocyte transformation skin test

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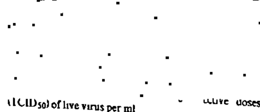
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Visna virus causes a slow infection in sheep which is characterized by a long subclinical period sometimes followed by a slowly progressing fatal disease The virus does not replicate in small laboratory animals and numerous early attempts to immunize rabbits and other rodents with visna virus were unsuccessful (15) However later experiments have shown that rabbits respond to hyperimmunization with concentrated virus by producing significant titers of precipitating complement fixing and sometimes neutralizing antibodies (4 9) More recently specific visna virus polypeptides have been identified as the most active antibody producing antigens in rabbits (8 10) In the present study we have tested the cell mediated immune (CMI) response in rabbits immunized with visna virus and have attempted to determine whether the visna virus polypeptides that are most active in the humoral immune response also play a major role in the C ...

MATERIALS AND METHODS

Virus growth and purification Strain K796 of visna virus was grown and assayed in cultures of sheep choroid plexus (SCP) cells maintained in Eagle basal medium supplemented with 2% lamb serum (1 15) Virus purification was done by the method of *Lin & Thormar* (7) except that sonication was omitted Purified virus resuspended in 0 15 M phosphate buffered saline (PBS) pH 7 2 was used in all experiments

Isolation and purification of visna virus proteins Visna polypeptides P25 P14 and P12 were purified by agarose gel filtration in 6M guanidine hydrochloride as descr...



(TCID50) of live virus per ml ... ctive doses

Immunization of rabbits New Zealand White male rabbits (2–3 kg) were immunized with a mixture of virus and complete Freund s adjuvant (CFA) (H 37 Ra Difco Laboratories Detroit Mich) The first injection was intradermal (id) into the hind footpads using about 100 µg of viral antigen (10^9 $TCID_{50}$ of live or inactivated virus) per animal A second injection containing CFA was given intramuscularly (im) into the hind legs 6–10 weeks later using about 50 µg of viral antigen ($10^{8.7}$ $TCID_{50}$ of virus) per animal Control animals were injected with SCP cell homogenate in maintenance medium mixed with CFA All rabbits were bled from the central ear artery at selected intervals before and after immunization

Immunodiffusion complement fixation (CF) and neutralization tests All of these tests were done as described earlier (9) A purified visna virus preparation was used as antigen in the immunodiffusion and CF tests and sera were thoroughly absorbed with lyophilized SCP cells in maintenance medium

Lymphocyte transformation (LT) test Peripheral heparinized blood was mixed with a solution of dextran T250 (Pharmacia Fine Chemicals Piscataway N J) and incubated at 37 °C for 30–45 min The leukocyte rich supernatants were centrifuged and the pellets resuspended in RPMI 1640 medium (Grand Island Biological Co) containing 25 mM of Hepes buffer glutamine and 20% autologous rabbit serum Unless otherwise stated the serum used was collected from each rabbit before immunization 2×10^5 cells in 0 1 ml of medium were dispensed into the wells of sterile Multi Dish Disposo Trays (Linbro Scientific Inc New Haven Conn) and 0 1 ml of mitogen or antigen in RPMI 1640 medium without serum was added to triplicate wells Concanavalin A (ConA Calbiochem LaJolla CA) 1 µg/well was used as mitogen Visna virus in 3–4 dilutions was used as antigen unless otherwise stated The amount of virus protein per culture ranged from 0 2–4 µg Dilutions of freeze thawed homogenate of uninfected SCP cells in maintenance medium served as control After incubation of the lymphocyte cultures in a humidified CO_2 incubator at 37 °C for 72 h 1 µCi of [3H] thymidine (59 Ci/mmol New England Nuclear Boston Mass) in 0 05 ml of medium was added to each well Sixteen hours later the cultures were harvested washed and counted in a Packard Tri Carb liquid scintillation spectrometer Model 544 The results are presented as stimulation indices (SI) obtained by dividing the mean cpm of triplicate cultures containing visna or control antigens by cpm of cultures containing growth medium alone

Skin test The animals were carefully shaved on the flank immediately lateral to the vertebral column and 0 1 ml volumes of viral and control antigens in PBS were injected intracutaneously at about 3 cm intervals Three 2 fold dilutions of each antigen were used ranging in protein content from 1 to 4 µg/0 1 ml The inoculation sites were examined at 24 48 and 72h after injection and the diameters of erythema measured

Histology Rabbits were sacrificed and the skin test sites fixed in 10% formalin and embedded in paraffin Sections were stained with hematoxylin-eosin

RESULTS

Stimulation of Lymphocytes by Live and UV Inactivated Virus

Rabbits were bled weekly for 6 weeks following immunization and their lymphocytes tested in LT test against viral and control antigens Data from 3 separate experiments are combined in Fig 1 which shows the lymphocyte stimulation in 4 rabbits injected with control antigen (A) 6 rabbits injected with uv inactivated virus (B C) and 9 rabbits injected with live virus (D E) The points represent the mean SI values of each group of animals It can be seen that visna virus sensitized lymphocytes appeared in the blood of rabbits already one week after immunization and showed maximal SI at about 2 weeks after immunization (B D) In the 9 rabbits immunized with live virus the maximum SI varied from 4 to 23 with a mean of 13 ± 6 (SD) Similarly in the 6 rabbits immunized with uv inactivated virus the SI ranged from 3 to 14 with a mean of 11 ± 4 At 3 weeks after immunization the SI decreased and reached insignificant levels at 4–5 weeks A booster given at 6–10 weeks resulted in a rapid resensitization of lymphocytes lasting for less than 3 weeks (C E) The maximum SI after the booster was similar to that following primary immunization and showed the same variation The stimulation by control antigen was negligible even

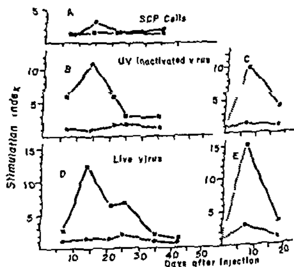


Fig 1 Cell mediated immune response to visna virus in immunized rabbits The first injection was id into footpads (A B D) followed by im injection (C E) Peripheral lymphocytes were tested at about weekly intervals in lymphocyte transformation test against live purified visna virus (■) and homogenized SCP cells (●) Lymphocytes from the control rabbits (A) did not respond to the viral antigen whereas there was a significant transient response in rabbits immunized with uv inactivated (B C) or live virus (D E)

he 4 rabbits immunized with freeze thawed p cell suspension in maintenance medium (3 *

Lymphocytes from these rabbits were not ulated by purified visna virus There was not a if cant difference in the lymphocyte response of its immunized with either live or uv inactivated s and both were equally active as antigen in the test (data not shown)

1 one experiment post immunization sera col d from 2 rabbits at the same time as phocytes were used in the LT test Although imal lymphocyte stimulation by viral antigen at weeks was up to twice as high in wells aining 10% post immunization sera as in wells aining 10% presera the SI decreased to tectable levels in both types of sera at 4–5 ks

yed Hypersensitivity

lost of the rabbits were not skin tested until 6–9 ks after the footpad injection Rabbits immuni with live or uv inactivated visna virus reacted to viral antigen by formation of skin erythema and



Fig 3 A section from the skin of a visna virus immunized rabbit fixed 5 days after testing with 1 µg of viral antigen Heavy infiltration of lymphocytes Hema toxylin-eosin × 166



: 2 Typical skin erythema in a visna virus munized rabbit 3 days following challenge with 4 2 d 1 µg respectively of live purified visna virus All of test s tes had a sign ficant induration The reaction to control antigen was no longer detectable Picture ows actual size

induration These rabbits responded to the control antigen by a slight erythema but there was no induration of the skin At 72 h the erythema caused by the control antigen had disappeared whereas the reaction to viral antigen was still distinct (Fig 2) In

antigen reacted to that antigen in the skin test whereas their reaction to the virus was much weaker and without induration Two rabbits which were skin tested 2 weeks after footpad injection with live virus showed a positive reaction to viral antigen

A histological examination of the skin reaction in 4 rabbits sacrificed 5 days after the skin test showed that visna virus immunized rabbits responded to the virus by heavy infiltration of small lymphocytes (Fig 3) whereas there was no detectable cellular reaction to the same amount of control antigen A rabbit immun zed

the other

in its skin

no reactio

Humoral Response

Rabbits immunized with either live or uv-inactivated visna virus formed CF and precipitating antibodies against purified virus 2–3 weeks after the first immunization, with CF titers of 1 4–1 32 The antibody activity was similar in both groups Neutralizing antibodies were not detectable until after the booster injection and then only in low titers (1 4–1 16) One rabbit that received three booster injections with live purified virus formed neutralizing antibodies with a titer of 1 2048

Response to Purified Visna Proteins P25 P14 and P12

Although sera from visna virus immunized rabbits reacted against purified visna protein P25 in the immunodiffusion test, their lymphocytes did not respond to this protein in the LT test In one rabbit immunized with uv-inactivated virus there was a slight reaction to P25 in the skin test, but much less than to the whole virus This was confirmed by histological examination of the skin test site which showed a moderate infiltration of inflammatory cells There was no reaction to purified proteins P14 and P12 in any of the tests

DISCUSSION

The lymphocyte response in rabbits immunized with purified visna virus is similar to that reported for a number of other viruses in laboratory animals (2, 3, 13, 14) Maximum sensitization occurred within 2 weeks and declined to control levels during the ensuing 2–3 weeks In contrast, a delayed type cutaneous hypersensitivity to the virus lasted for several weeks after immunization There is therefore a lack of correlation between these two reactions in visna virus immunized rabbits Since it has been reported that antigen antibody complexes enhance the stimulation of sensitized lymphocytes from immunized animals (12) we used post immunization sera in one LT experiment to see if a better correlation with the skin test would be obtained These sera contained precipitating visna virus antibodies but were free of neutralizing antibodies The presence of the immune sera was found to enhance lymphocyte stimulation during the peak response However, there was no significant lymphocyte stimulation in the presence of varying concentrations of immune sera at 8 weeks after immunization when the skin reaction was still positive

We were not successful in identifying visna virus proteins that are active in stimulating the CMI response in rabbits In a previous study (8) sera from rabbits immunized with visna virus were found to precipitate 18 different viral polypeptides The strongest reaction was against polypeptides P25, P14 and P12 However, none of them stimulated the lymphocytes of immunized rabbits and only P25 was slightly active in the skin test These individual proteins are either not the major stimulants of the cellular immune system or the highly purified preparation used in the LT and skin tests had lost the specific antigenic activity More studies are therefore needed to determine which viral components, i e proteins, glycoproteins or lipids, elicit the cellular immune response either individually or in combination

We have consistently failed to detect lymphocyte stimulation in sheep persistently infected with visna virus and skin tests have also been negative However, a lasting delayed type hypersensitivity to visna virus can be elicited in sheep by id injection of concentrated virus in CFA (*Thomar* unpublished data) If the CNS lesions in visna are caused by a chronic immune reaction to viral antigens (11) immunization of persistently infected sheep with visna virus in CFA can be expected to enhance the lesions, depending on the quantity and distribution of viral antigen This procedure should therefore result in an increased number of infected sheep showing clinical signs of visna In view of the similar immune responses in rabbits and sheep injected with visna virus in CFA and the difficulties involved in working with a large number of sheep we suggest that rabbits may be used as a model to study the CMI reaction to visna virus antigens in the brain and other organs (5) However, unless a persistent visna virus infection can be established in the rabbit the effect of enhanced CMI reactions on the pathogenesis of the visna disease must eventually be studied in sheep

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EFFECT OF IMMUNOSPECIFIC Fab FRAGMENTS OF ANTIRENIN ON PLASMA RENIN AND BLOOD PRESSURE

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Lykkegård S Effect of immunospecific Fab fragments of antirenin on plasma renin and blood pressure Acta path microbiol scand Sect C 88 179–185 1980

Anti mouse renin specific Fab fragment was labelled with ^{125}I and repurified on an affinity-column with renin as ligand in order to eliminate Fab fragments with a damaged antibody combining site The ^{125}I labelled Fab fragment, with addition of unlabelled Fab fragment was administered *in vivo* to sialo-adenectomized mice The serum disappearance curve of the Fab fragments with the intact antibody combining site possessed two components with a half life of 120 min for the slowest component The renin specific Fab fragments inhibited the enzymatic activity of renin with a K_I value of $5\ 5 \pm 1\ 5 \times 10^{-9}$ M compatible with competitive inhibition The same Fab fragments were able to inhibit 99% of the enzymatic active renin The administration of the Fab fragments caused a 3–4 fold increase in the total concentration of plasma renin but the enzymatic activity was inhibited No decrease in blood pressure was demonstrated in the animals

Key words Renin inhibition Fab fragment pharmacokinetic blood pressure

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Inhibitors of the renin angiotensin system have made it possible to define the role of renin in both physiologic and pathologic situations and to under stand the response of the renin angiotensin system to a variety of interventions The inhibitors interfere with the action of renin on plasma renin substrate blocking converting enzyme in its action on angiotensin I or compete with angiotensin II at its receptor site

The purification to homogeneity of renin from the submaxillary glands of mice (5 2) rendered it possible to raise antibodies which inhibit the enzymatic activity of renin and block the action of renin on plasma renin substrate Renin specific Fab fragments prepared from these antibodies were characterized in vitro (11), as to their inhibitory effect on the enzymatic activity of renin The present paper deals with the effect of the inhibitory Fab fragments on the renin activity and the blood pressure after intraarteriel administration in mice The pharmacokinetic of Fab fragments was studied by measuring the rate of clearance of radiolabelled Fab fragments from the blood

MATERIAL AND METHODS

Animals Albino male mice of Danish Serum Institute strain weighing about 50 g and containing high concentrations of renin in the submaxillaris glands were used (3) All animals were sialo-adenectomized 14–30 days before the experiments A catheter was inserted into one or both femoral arteries during a short term ether anaesthesia After the operation the mice were placed in restraining cages The mice were conscious during the rest of the experiment. When recording the blood pressure a Tybjærg Hansen transducer and a Servogor 511 recorder was used

Rabbit anti mouse renin specific Fab fragments were prepared according to *Lykkegård* (11) from rabbits immunized with pure mouse submaxillary renin (5 12)

RESULTS

The Serum Disappearance Rate of Renin Specific ¹²⁵I labelled Fab fragments

4.7×10^{-13} mol of repurified ^{125}I labelled Fab fragment with intact antibody combining site was given as a single bolus i a. to a sialo adenectomized conscious mouse The serum disappearance curve of Fab fragments (Fig 1) had two components with a $t_{½}$ values of 30 min and 120 min In order to evaluate the amount of free ^{125}I and *in vivo* degraded ^{125}I labelled Fab fragments in the serum samples Fab fragments were precipitated with anti rabbit IgG antibodies directed against light and heavy chains As seen in (Fig 1) the serum disappearance curves for the total counts and the precipitated ^{125}I labelled Fab fragments were almost identical indicating that the disappearance curve represented the disappearance rate for intact ^{125}I labelled Fab fragments The addition of unlabelled Fab fragments to ^{125}I labelled Fab fragments did not alter the serum disappearance curve for the radiolabelled Fab fragments

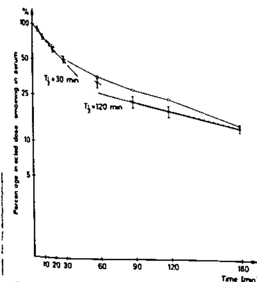


Fig 1 Serum disappearance curves of rabbit anti mouse renin Fab fragments in sialo-adenectomized mice The data are expressed as percentage of injected material remaining in serum versus time in minutes The dotted line indicates direct measurements of ^{125}I counts while the solid line indicates determination of ^{125}I after precipitation with antirabbit IgG (light and heavy chains) followed by extensive washing (mean of 3 experiment)

Quantitative Analysis of the Interaction of Renin Specific Fab Fragments and Mouse Submaxillary Renin in vitro

The inhibition kinetics of Fab fragments *in vitro* was studied in order to determine the Michaelis constant (K_m) the inhibitor constant (K_I) and rate constant (k_2) values for Fab fragment as a competitive inhibitor of renin and to use them in the evaluation of the effect of Fab fragments on renin *in vivo* The K_m and K_I values were determined by using varying concentrations of rat renin substrate (S) and fixed concentrations of Fab fragments (I) Lineweaver Burk plots of the enzymatic inhibition by Fab fragments are presented in (Fig 2) with three different concentrations of Fab fragments The data are compatible with competitive inhibition The plot gives a K_m value of 1.79 ± 0.10 μM and a maximum velocity (V_{max}) of $3.75\pm0.21\times10^{-8}$ $M\times h^{-1}$ The estimate of the inhibitor constant (K_I) is $5.5\pm1.5\times10^{-9}$ M The calculation of K_I was made from the equation for competitive inhibition (14)

$$v = \frac{V_{max}}{1+\frac{K_m}{[S]}\left(1+\frac{[I]}{K_I}\right)}$$

From equation [1] V_{max} being $k_2\times E_o$, a k_2-value of 6.8×10^{5} $M\times h^{-1}\times GU^{-1}\times ml$ was [illegible]

and the homologous substrate was performed with substrate concentrations below the Michaelis constant and the K_m value was greater than 1.79 μM

The Effect of Anti mouse Renin Specific Fab Fragment on the Activity of Renin in vivo

Fab fragments were injected i a to sialo adenectomized mice The plasma samples drawn at different periods of time after *in vivo* administration of Fab fragments were incubated with varying concentrations of rat renin substrate as described in Material and Methods The rate of generation of angiotensin I and the initial velocities as a function of substrate concentration were presented in a Lineweaver Burk plot (Fig 3)

The total concentration of plasma renin was determined from the intersection on the ordinate as described in Material and Methods The values obtained are shown as a function of time in Fig 4 lower panel The renin concentration rose to a 3–4 fold higher level than before injection

The Fab fragment concentration in plasma as a function of time was calculated from the serum disappearance curve of Fab fragment (Fig 1) The values are presented in Table I column B The Fab

^{125}I-labelled rabbit anti-mouse renin specific Fab fragments were prepared by using 5 µg Fab fragments (Extinction coefficient at 280 nm

$$[E^{1\%}_{1\ cm} = 15\ 3](10),$$

in 50 µl 20 mM citrate buffer pH 3 0 Twenty-five µl 0 5 M phosphate buffer pH 7 5 was added to 100 µCi of carrierfree ^{125}I (Amersham IMS 30) contained in 10 µl

Fab fragments and radioactive iodide were mixed just before the addition of 25 µl 12 mM chloramine-T freshly dissolved in 50 mM phosphate buffer pH 7 5 (9) The reaction time was 30 s with constant mixing, followed by the addition of 100 µl 12 mM sodium metabisulfite Finally 100 µl 60 mM potassium iodide containing 1 % human albumin was added and the mixture transferred to 0 5 × 30 cm column of Sephadex G-25 fine (Pharmacia) precoated with albumin The labelled Fab fragments were eluted as a single peak which was diluted with 50 mM phosphate buffer pH 7 5 containing 0 5% human albumin and frozen immediately in small aliquots The specific activity was calculated to be 11 6 µCi/µg or approximately 1 iodine atom per 10 molecules of Fab fragments

Purification of ^{125}I-labelled Fab fragments by affinity chromatography Just before use the labelled Fab fragments were applied to an affinity column, activated CH-Sepharose 4M (Pharmacia) with pure submaxillary renin as a ligand (11), in order to exclude the Fab fragments which had been damaged at the antibody combining site during the labelling procedure After extensive washing of the column with 20 mM phosphate buffer pH 7 2 containing 1 M NaCl, the labelled Fab fragments with undamaged antibody combining site were eluted with 20 mM citrate buffer pH 3 0 with a recovery of 17–18% The peak sample was dialyzed extensively against distilled water before being lyophilized The labelled Fab fragments were dissolved in 0 15 M NaCl containing 0 1% pure human albumin (Behringwerke)

Blood sampling Blood samples were drawn from the femoral artery in a volume of approximately 30 µl at 5 10, 20, 30, 60, 90, 120 and 180 min after injection of ^{125}I-labelled Fab fragments of various specific activity After centrifugation the samples were stored at −20 °C until analyzed

Measurements of plasma Fab fragments The direct determination of Fab fragments in plasma was carried out by measuring the ^{125}I counts in 5 µl samples diluted in 100 µl 50 mM phosphate buffer pH 7 5, containing 0 5% human albumin and a known small amount of normal rabbit plasma as carrier IgG After counting the samples 50 µl hog antirabbit IgG (light and heavy chains) (DAKO) of an appropriate dilution was added in order to specifically precipitate ^{125}I labelled Fab fragments The samples were incubated 24 h at 4 °C After incubation 2 0 ml 50 mM phosphate buffer pH 7 5 was added to the tubes and they were centrifuged at 3000 *g* for 30 min The supernatant was removed except for 200 µl containing the precipitate The washing step was repeated three times and the radioactivity in the precipitate was counted The *indirect determination* of Fab fragments from the kinetic data was performed as described below

Plasma renin concentrations Enzymatic active renin was determined by the antibody trapping method (15) in which angiotensin I formed by the catalytic activity of renin was captured and measured by radioimmunologic technique

Determination of the inhibitor constant (K_I) The K_m value for the reaction between pure submaxillary mouse renin and rat or mouse renin substrate was determined as described by *Poulsen & Jørgensen* (15) Twenty µl aliquots of a serial dilution of renin substrate were incubated at

0 5 × 10^{-3}

and 5 µl of

albumin When *the inhibitor constant* K_I was determined, the 5 µl Tris buffer was replaced with 5 µl of a Fab fragment concentration of 3 1–12 3 × 10^{-9} M For determination of the inhibitory effect of Fab fragments in mouse plasma, the 5 µl pure submaxillary renin was also omitted and 5 µl of plasma samples diluted in 0 1 M Tris-HCl (pH 7 5) containing 0 5% albumin was used

The inhibitor constant (K_I) and the Michaelis constant (K_m) were obtained from a weighted least squares fit of a Lineweaver-Burk plot Each point was given a weight proportional to the initial velocity (7)

The indirect determination of the Fab fragment and renin concentration in plasma After *in vivo* administration of anti-mouse renin specific Fab fragments plasma samples were drawn at different period of time The samples, containing both renin and Fab fragments, were incubated with a serial dilution of rat renin substrate under essentially the same conditions as above The rates of generation or initial velocities as a function of substrate concentration were fitted to a Lineweaver Burk plot (Fig 3) Assuming competitive inhibition (6) the maximum velocity (V_{max}) was calculated from the point of intersection on the ordinates From the equation of V_{max} being $k_2 \times E_o$ [1] the renin concentrations were calculated, using the k_2-value determined by using known renin (E_o) values (Fig 2) From the intersection on the abcissa, corresponding to

$$-\frac{1}{K_m\left(1 + \frac{[I]}{K_I}\right)} \quad [2]$$

the inhibitor concentration (I) was calculated (Fig 3) by using K_m- and K_I-values as determined above

The degree of enzymatic inhibition of renin in vivo The concentration of renin Fab fragments and the K_I value are used to determine the degree of enzymatic inhibition of renin *in vivo* as follows The reaction between renin and Fab fragment meets the requirements of the law of mass action

$$\frac{x}{Ag_o - x} = K_a(Ab_o - x) [3]$$

Ag_o being the total renin concentration Ab_o being the total concentration of Fab fragments x being the renin Fab fragment complex and K_a being the inverse K_I value The left side of equation [3] is the ratio between bound and free antigen concentration at equilibrium from which the percentage of inhibition can be calculated

RESULTS

The Serum Disappearance Rate of Renin Specific ^{125}I labelled Fab fragments

4.7×10^{-13} mol of repurified ^{125}I labelled Fab fragment with intact antibody combining site was given as a single bolus i.a. to a sialo-adenectomized conscious mouse. The serum disappearance curve of Fab fragments (Fig. 1) had two components with a $t_{1/2}$ values of 30 min and 120 min. In order to evaluate the amount of free ^{125}I and *in vivo* degraded ^{125}I labelled Fab fragments in the serum samples Fab fragments were precipitated with anti-rabbit IgG antibodies directed against light and heavy chains. As seen in (Fig. 1) the serum disappearance curves for the total counts and the precipitated ^{125}I labelled Fab fragments were almost identical indicating that the disappearance curve represented the disappearance rate for intact ^{125}I labelled Fab fragments. The addition of unlabelled Fab fragments to ^{125}I labelled Fab fragments did not alter the serum disappearance curve for the radiolabelled Fab fragments.

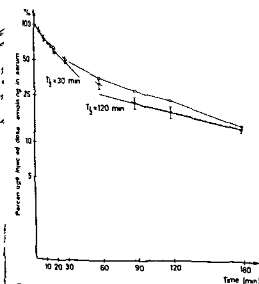


Fig. 1. Serum disappearance curves of rabbit anti mouse renin Fab fragments in sialo-adenectomized mice. The data are expressed as percentage of injected material remaining in serum versus time in minutes. The dotted line indicates direct measurements of ^{125}I counts while the solid line indicates determination of ^{125}I after precipitation with antirabbit IgG (light and heavy chains) followed by extensive washing (mean of 3 experiment).

Quantitative Analysis of the Interaction of Renin Specific Fab Fragments and Mouse Submaxillary Renin in vitro

The inhibition kinetics of Fab fragments *in vitro* was studied in order to determine the Michaelis constant (K_m), the inhibitor constant (K_I) and rate constant (k_2) values for Fab fragment as a competitive inhibitor of renin and to use them in the evaluation of the effect of Fab fragments on renin *in vivo*. The K_m and K_I values were determined by using varying concentrations of rat renin substrate (S) and fixed concentrations of Fab fragments (I). Lineweaver Burk plots of the enzymatic inhibition by Fab fragments are presented in (Fig. 2) with three different concentrations of Fab fragments. The data are compatible with competitive inhibition. The plot gives a K_m value of 1.79 ± 0.10 µM and a maximum velocity (V_{max}) of $3.75 \pm 0.21 \times 10^{-8}$ $M \times h^{-1}$. The estimate of the inhibitor constant (K_I) is $5.5 \pm 1.5 \times 10^{-9}$ M. The calculation of K_I was made from the equation for competitive inhibition (14)

$$v = \frac{V_{max}}{1 + \frac{K_m}{[S]}\left(1 + \frac{[I]}{K_I}\right)}$$

From equation [1] V_{max} being $k_2 \times E_o$ a k_2-value of 6.8×10^{-5} $M \times h^{-1} \times GU^{-1} \times ml$ was calculated from the known concentration of renin and the intercept on the ordinate ($\frac{1}{V_{max}}$).

The reaction between mouse submaxillary renin and the homologous substrate was performed with substrate concentrations below the Michaelis constant and the K_m value was greater than 1.79 µM.

The Effect of Anti mouse Renin Specific Fab Fragment on the Activity of Renin in vivo

Fab fragments were injected i.a. to sialo-adenectomized m... Th...

...concentrations of rat renin substrate as described in Material and Methods. The rate of generation of angiotensin I and the initial velocities as a function of substrate concentration were presented in a Lineweaver Burk plot (Fig. 3).

The total concentration of plasma renin was determined from the intersection on the ordinate as described in Material and Methods. The values obtained are shown as a function of time in Fig. 4 lower panel. The renin concentration rose to a 3–4 fold higher level than before injection.

The Fab fragment concentration in plasma as a function of time was calculated from the serum disappearance curve of Fab fragment (Fig. 1). The values are presented in Table 1 column B. The Fab

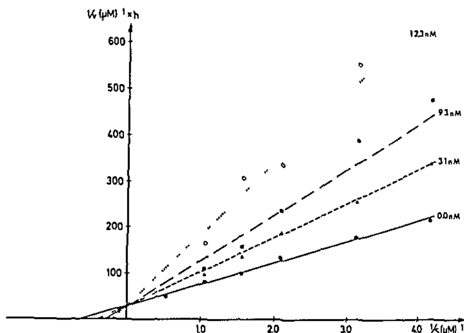


Fig 2 A Lineweaver Burk plot of the reaction between submaxillary renin and rat renin substrate in the presence of varying concentrations of rabbit anti mouse renin specific Fab fragments The concentrations of renin was $5\,0 \times 10^{-4}$ GU/ml and the incubation time was 1 0 h at 37 °C, pH 7 5 The concentration of Fab fragments was 0 0 $3\,1 \times 10^{-9}$ *M*, $9\,3 \times 10^{-9}$ *M and* $12\,3 \times 10^{-9}$ *M*

fragment concentration was also determined directly from the intersection on the abcissa as described in Material and Methods Only the initial value was statistically significant to the value obtained before administration of Fab fragments (Table 1 column C)

The association constant (K_a) for the reaction between submaxillary renin and Fab fragment was

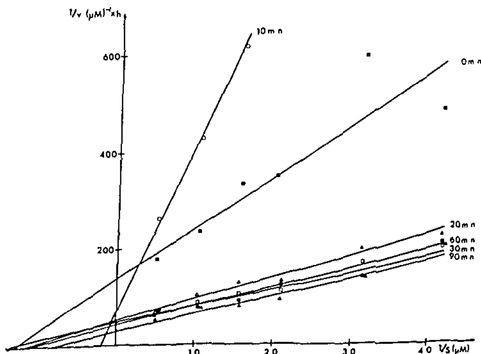


Fig 3 A Lineweaver-Burk plot of the plasma samples from the sialo adenectomized mice and the rat renin subst[illegible] The samples were obtained at different times after intraarterial administration of renin specific Fab fragm[illegible] 37 °C and pH 7 5

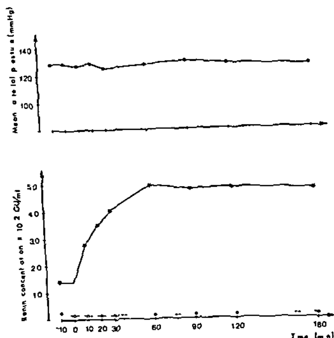


Fig 4 The upper panel shows the mean arterial blood pressure after intraarterial administration of 1 8 × 10^{-9} mol renin specific Fab fragment The lower panel shows the corresponding renin concentration in plasma The renin concentration is the mean of 3 experiments for the sialoadenectomized mice (★) and a single determination for the sialo adenectomized and nephrectomized mouse (O)

determined as the inverse of the K_I value (see above)

The percent inhibition of plasma renin could now be determined as described in Material and Methods from the total concentration of plasma renin the concentration of the Fab fragment and the association constant (Table 1 column A) The percentage of renin in mouse plasma which was bound and enzymatically inhibited by Fab fragments was more than 99% initially Even after 2 hours 98% was inhibited

The blood pressure responses are shown in Fig 4 The blood pressure was uninfluenced by administration of Fab fragments (n = 5) The rise of 3–4 times in the concentration of renin (Fig 4) was in accordance with an effect on the direct negative feedback of angiotensin II on renin secretion (8) Even after this rise in the concentration of renin the enzymatic activity was totally inhibited (Table 1) In a similar experiment with nephrectomized mice the renin concentration was 8 0 × 10^{-4} GU/ml and remained at the same low level after administration

TABLE 1 *The Concentration of Fab Fragment in Plasma and the Corresponding Renin Activity Inhibited*

Time (min)	A Renin activity inhibited in plasma	The Fab fragment conc in plasma B Data from Fig 1	C Data from Fig 3
10	99 4%	7 5 × 10^{-7} M	3 1 ± 0 4 × 10^{-7} M
20	99 3%	6 0 × 10^{-7} M	–
30	99 2%	4 8 × 10^{-7} M	–
60	98 8%	2 9 × 10^{-7} M	–
90	98 2%	2 0 × 10^{-7} M	–
120	97 9%	1 7 × 10^{-7} M	–

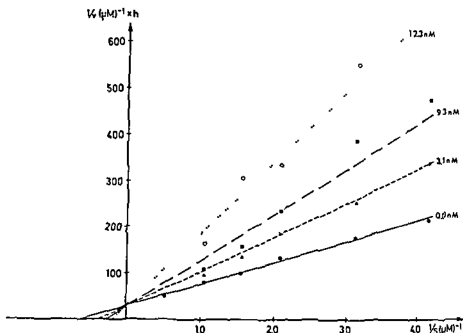


Fig 2 A Lineweaver-Burk plot of the reaction between submaxillary renin and rat renin substrate in the presence of varying concentrations of rabbit anti-mouse renin specific Fab fragments The concentrations of renin was $5\,0\times10^{-4}$ GU/ml and the incubation time was 1 0 h at 37 °C, pH 7 5 The concentration of Fab fragments was 0 0, $3\,1\times10^{-9}$ M, $9\,3\times10^{-9}$ M and $12\,3\times10^{-9}$ M

fragment concentration was also determined directly from the intersection on the abcissa as described in Material and Methods Only the initial value was statistically significant to the value obtained before administration of Fab fragments (Table 1 column C)

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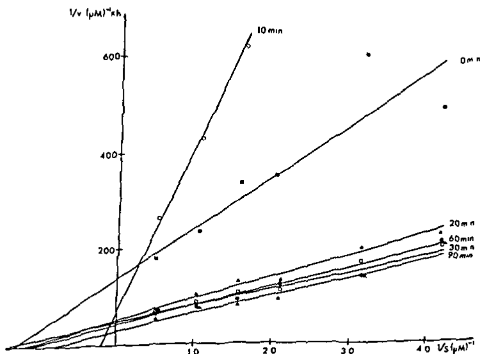


Fig 3 A Lineweaver-Burk plot of the plasma samples, from the sialo-adenectomized mice, and the rat renin subst[illegible] The samples were obtained at different times after intraarterial administration of renin specific Fab fragn[illegible] 37 °C and pH 7 5

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of Fab fragments In both cases the blood pressure remained stable during the test period, as indicated in Fig 4

DISCUSSION

The *in vivo* administration of purified Fab fragments directed against the mouse renin, has permitted us to study the role, the renin angiotensin system plays in vascular homeostasis in mice The participation of the renin-angiotensin system in the homeostasis of blood pressure in mice has been studied previously by *Bing & Poulsen* (4) by blockade of the renin system in order to see whether both renal and submaxillary renin participated in the homeostasis The study was performed with infusion of a competitive angiotensin II inhibitor (Saralasin) or an inhibitor of angiotensin I converting enzyme (SA 20 881) and the results clearly indicated that only renal renin participated in the homeostasis The serum disappearance rate curve of the repurified ^{125}I-labelled Fab fragment, with or without the addition of unlabelled Fab fragment, possessed two components with $t_{½}$ of 120 min for the slowest component Previous studies of the disappearance rate of homologous and heterologous immunoglobulin fragments from serum in rats (1, 16) showed a halflife for the Fab fragments of less than 60 min and 102 min respectively Studies by *Wochner et al* (17) of the disappearance rate for rabbit Fab fragments in mice were performed as a whole body catabolic disappearance rate and showed a halflife of 216 min for the Fab fragments The discrepancy between the halflife of 216 min found by *Wochner et al* (17) and the halflife of 120 min (Fig 1) may be explained by the distribution of Fab fragments in an extravascular pool

The interaction of enzymes with their specific antibodies frequently causes inhibition of function by either noncompetitive (2) or competitive (13) mechanisms The Lineweaver Burk plot in Fig 2 is in agreement with a competitive inhibition with an inhitor constant (K_I) of $5\,5 \pm 1\,5 \times 10^{-9}$ M, indicating that the affinity of the Fab fragment ($\frac{1}{K_I}$) for a site required for the enzymatic activity of renin is two orders of a magnitude higher than the Michaelis constant (K_m) for the reaction between renin and its homologous substrate

The calculation of the percentage of renin which is inhibited by Fab fragments, is based on the assumption that the kinetic constant of the reaction of plasma renin and submaxillary renin were the same Since plasma renin was not pure and was found together with varying concentration of homologous substrate, the K_a-value for plasma renin could not be determined directly However, in Table 1, the Fab fragment concentration in column B and C was similar, and column B was calculated with the assumption that the K_a-values were similar for plasma renin and pure submaxillary renin This indicated similarity in K_a-values for plasma and pure submaxillary renin The 3–4 fold increase in the total concentration of renin (though bound) in plasma after the Fab fragment administration as seen in Fig 4, is probably a result of two parameters Firstly, an accumulation of secreted renin and secondly a result of a blocked negative feed-back mechanism on renin secretion As indicated in Table 1 column A, the Fab fragment concentration after 60 min was still able to inhibit the renin activity completely In order to see whether or not this interference of the negative feed back mechanism with Fab fragment was due to renal renin, the Fab fragment was also used in nephrectomized mice The result as indicated in (Fig 4) showed no rise in plasma renin concentration The blood pressure was the same before and after administration of Fab fragments

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BACTERIA ASSOCIATED WITH ACUTE OTITIS MEDIA HAVE HIGH Cl_q BINDING CAPACITY

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Prellner K Bacteria associated with acute otitis media have high Cl_q binding capacity Acta path microbiol scand Sect. C 88 187 190 1980

A simple rapid and sensitive radioimmunologic method for demonstrating Cl_q binding to bacteria is described Various bacteria were shown to bind Cl_q without the participation of antibodies Great differences in Cl_q binding levels between different types and strains of *S pneumoniae* were found A high uptake of Cl_q was observed for many pneumococcal strains of type VI XIX and XXIII and also for non typable strains of *H influenzae* and *B catarrhalis* Other bacteria tested including *H influenzae* types a–f demonstrated less capacity to bind Cl_q

Key words Complement Cl_q-binding substances pneumococci haemophilus branhamella

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Received 5 xi 79 Accepted 12 ii 80

Decreased serum levels of Cl_q with concomitant normal or elevated levels of Clr and Cls are found in many patients with otitis media (2 7) Recently we have reported Cl_q binding substances in the sera and middle ear effusions of such patients (7) The nature of these Cl_q binding substances have not been recognized but they may represent complexes of antibodies and bacterial antigens *Loos et al* (8) have shown that the first component of complement, Cl binds to bacterial lipopolysaccharides (LPS) without the presence of antibodies and they also demonstrated that intact bacteria such as *E coli* and *K pneumoniae* had this capacity (9)

In the present study a method to quantitate the uptake of Cl_q by intact bacteria was designed acterial species known to cause acute otitis media children were investigated regarding their capacity to bind Cl_q independently of the presence of ntibodies

MATERIAL AND METHODS

acterial Strains and Suspensions

Eighty-seven strains were obtained from clinical specimens sent to the Department of Medical Microbiology University Hospital Lund for bacteriological examination The bacteria were 37 strains of *S pneumoniae* of which 3 were type I 4 were type III 4 were type VI 4 were type XIV 4 were type XVIII 13 were type XIX and 5 were type XXIII 22 strains of *H influenzae* of which 2 were type a 7 were type b 3 were type c 3 were type d 3 were type f and 4 were non typable *B catarrhalis* 7 strains *E coli* 4 *S aureus* 4 *S epidermidis* 2 *S saprophyticus* 2 group A streptococcus 1 group B streptococci 2 group C streptococci 2 and *S faecalis* 2 strains

S pneumoniae H influenzae and *E coli* were identified according to conventional methods The pneumococcal serotypes were determined by the Quellung reaction of *Neufeld* (10) *H influenzae* were typed by slide agglutination *B catarrhalis* was identified as described by *Kamme* (3) The *S aureus S epidermidis* and S saproph... tional (13) *S fae* *H*

... bacteria were washed three times in sucrose veronal buffer (SVB)· 0 27 moles sucrose 25 mmoles sodium barbital 0 1 mmol Ca^{++} and 0 5

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BACTERIA ASSOCIATED WITH ACUTE OTITIS MEDIA HAVE HIGH $C1_q$ BINDING CAPACITY

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Prellner K Bacteria associated with acute otitis media have high $C1_q$ binding capacity Acta path microbiol scand Sect C 88 187–190 1980

A simple rapid and sensitive radioimmunologic method for demonstrating $C1_q$ binding to bacteria is described Various bacteria were shown to bind $C1_q$ without the participation of antibodies Great differences in $C1_q$ binding levels between different types and strains of *S pneumoniae* were found A high uptake of $C1_q$ was observed for many pneumococcal strains of type VI XIX and XXIII and also for non typable strains of *H influenzae* and *B catarrhalis* Other bacteria tested including *H influenzae* types a–f demonstrated less capacity to bind $C1_q$

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Decreased serum levels of $C1_q$ with concomitant normal or elevated levels of C1r and C1s are found in many patients with otitis media (2 7) Recently we have reported $C1_q$ binding substances in the sera and middle ear effusions of such patients (7) The nature of these $C1_q$ binding substances have not been recognized but they may represent complexes of antibodies and bacterial antigens *Loos et al* (8) have shown that the first component of comple ment C1 binds to bacterial lipopolysaccharides (LPS) without the presence of antibodies and they also demonstrated that intact bacteria such as *E coli* and *K pneumoniae* had this capacity (9)

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[illegible]

H influenzae strains and *B catarrhalis* strains were obtained from hematin agar plates incubated at 37 °C for 18 hours in 6% CO_2 athmosphere The other strains were incubated in 20 ml Todd Hewitt broth at 37 °C for 18 hours The bacteria were washed three times in sucrose veronal buffer (SVB) 0 27 moles sucrose 25 mmoles sodium barbital 0 1 mmol Ca^{++} and 0 5

mmol Mg^{++} per liter pH 7 2 The bacterial suspensions were adjusted to A_{540} = 0 70 by opacity determinations The suspensions were concentrated ten times by centrifugation before use in the $C1_q$ binding assay

$C1_q$

$C1_q$ was purified from fresh human serum according to *Volanakis & Stroud* (14) The purified $C1_q$ was labelled with ^{125}I as described by *Sobel et al* (12) and stored at −80 °C ^{125}I $C1_q$ 0 3 mg/ml in 0 1 M phosphate buffer pH 7 0 was centrifuged at 13 000 *g* for 5 min in a Beckman microfuge before each experiment

Estimation of the Capacity of Bacteria to Bind $C1_q$

Principally the procedure described by *Sobel et al* (12) for binding of $C1_q$ to sensitized sheep cells (EA) was followed 200 µl of SVB 100 µl of the bacterial suspension to be tested and 10 µl of ^{125}I $C1_q$ was mixed Two hundred µl of the mixture was layered on 150 µl of a 40% sucrose solution in 0 05 M sodium phosphate buffer pH 7 0 in a 400 µl polyethylene microfuge tube (Beckman Instruments Inc Clinical Instrument Division Fullerton Calif) After centrifugation at 13 000 *g* for 15 min the tube was clamped with a hemostat above the sedimented bacteria and the tip cut off The radioactivity in the pellet and the supernatant was measured in an LKB Wallac 1270 Rackgamma gamma radiation counter (A B Biotec Stockholm Sweden) The $C1_q$ uptake by the bacterial sediment was calculated as a percentage of the radioactivity of the total $C1_q$ added

RESULTS

Methodological Investigation

Incubation temperature The uptake of ^{125}I labelled $C1_q$ by different strains of pneumococci and *E coli* was tested at 0 °C 20 °C and 37 °C maintaining the incubation time at 20 min and the conductance of the buffer at 7 mmho/cm Increasing the temperature from 0 °C to 20 °C increased the $C1_q$ uptake by *S pneumoniae* type XIX from 35 to 72 per cent and by *E coli* from 22 to 38 per cent At 37 °C the $C1_q$ uptake was increased further to 80 and 50 per cent respectively (Fig 1) For practical reasons an incubation temperature of 20 °C was used in the following experiments

Incubation time The $C1_q$ uptake at 20 °C was determined after incubation of the reagent mixture for 0 5 10 20 30 60 and 120 min respectively The conductance of the buffer used was 7 mmho/cm Ninety per cent of the total amount of $C1_q$ bound during 120 min incubation was taken up within the first 10 min This result was obtained with all strains tested (*S pneumoniae* type VI XIV XVIII XIX and *E coli*) The capacity of *S pneumoniae* type XIX to bind $C1_q$ at various

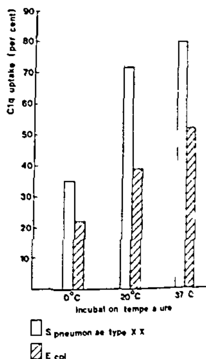


Fig 1 Binding of ^{125}I $C1_q$ to *S pneumoniae* type XIX and to *E coli* at different incubation temperatures

incubation times is shown in Fig 2 An incubation time of 20 min was used in the following studies

Conductance The effect of varying the conductance of the SV buffer used in the experiments was investigated $C1_q$ uptake was determined for one strain of each *S pneumoniae* type VI XIV XIX XVIII and one *E coli* strain The highest level of $C1_q$ binding was obtained between 7 and 9 mmho/cm Further increase of the conductance diminished the $C1_q$ uptake on the bacteria as illustrated with *S pneumoniae* type XVIII in Fig 3 In the following experiments a conductance of 7 mmho/cm was used

Variation in the values for uptake of $C1_q$ The uptake of $C1_q$ was measured for one strain of each *S pneumoniae* type III and type XIX cultured in 20 *ml Todd Hewitt broth* at 5 and 13 different occasions respectively using one $C1_q$ preparation The $C1_q$ uptake was expressed as a percentage mean ± 1SD and was for type III 9 9 ± 3 8 and for type XIX 76 8 ± 4 8

When different batches of $C1_q$ were used the numerical values for $C1_q$ uptake differed For one strain of *S pneumoniae* (type XIX) $C1_q$ uptake varied from 59 to 80 per cent However the relation between the $C1_q$ uptake for different bacterial strains was not influenced by the $C1_q$ preparation used

In control experiments unlabelled $C1_q$ was found to inhibit the uptake of radio labelled $C1_q$

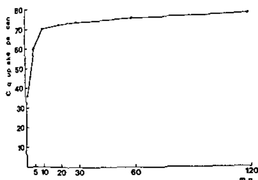


Fig 2 Kinetic analysis of ^{125}I $C1_q$ uptake by *S pneumoniae* type XIX

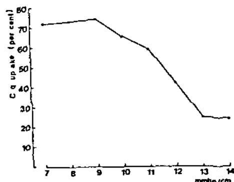


Fig 3 Binding of ^{125}I $C1_q$ to *S pneumoniae* type XXIII as a function of the conductance of the buffer

Binding of $C1_q$ to Different Bacteria

The results of $C1_q$ binding of various types of pneumococci and *H influenzae* as well as of *B catarrhalis* and *E coli* are presented in Fig 4

Non typable *H influenzae* had the highest $C1_q$ binding capacity varying between 87 and 92 per cent for the four strains tested *H influenzae* type a b c, d and f demonstrated less $C1_q$ binding and greater variation between different strains (5–55 per cent) All strains of *B catarrhalis* produced high and constant $C1_q$ binding values (62 to 76 per cent)

The $C1_q$ uptake of the different *S pneumoniae* types varied All type III strains tested gave low values (5 to 14 per cent) Marked variation were noted between different strains of *S pneumoniae* type I VI XIV XVIII XIX and XXIII A total of twenty strains of type VI XIX and XXIII were tested and eleven of these demonstrated $C1_q$ binding levels greater than 60 per cent

Four strains of *E coli* tested demonstrated $C1_q$ uptake of between 31 and 40 per cent

Streptococcus group A B C G and *S faecalis* bound $C1_q$ at levels between 27 and 55 per cent. The $C1_q$ uptake for four strains of *S aureus* was between 34 and 54 per cent. Two strains each of *S saprophyticus* and *S epidermidis* tested produced $C1_q$ binding levels about 20 and 35 per cent respectively

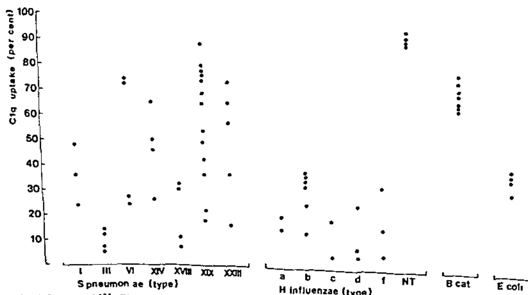


Fig 4 Binding of ^{125}I $C1_q$ to bacteria at 20 °C 20 min incubation time and the conductance of the buffer beeing 7 mmho/cm

DISCUSSION

The method described here for demonstrating Cl_q binding to bacteria is simple rapid and sensitive This study presents the capacity of various bacteria to bind Cl_q without the participation of antibodies

Our results concerning Cl_q uptake by *E coli* were in accordance with the findings of *Loos et al* (9) Furthermore we found that other bacteria gram positive and gram negative demonstrated even higher Cl_q binding

In acute purulent otitis media *S pneumoniae* is the most common causative agent followed by *H influenzae* and *B catarrhalis* (6) Among pneumococci we found a great difference in Cl_q binding levels between the different types and strains A high degree of Cl_q uptake was observed for many strains of types VI XIX and XXIII which are the serotypes most often isolated in pneumococcal otitis in young children (4) Otitis media caused by *H influenzae* is in 85 per cent provided for by non typable strains (5) In this investigation a striking difference in Cl_q uptake was demonstrated between *H influenzae* type a b c d f and the non typable strains The Cl_q uptake with typable *H influenzae* strains varied from 5 to 55 per cent while non typable strains produced values of about 90 per cent A high binding capacity (approximately 70 per cent) was also demonstrated with different strains of *B catarrhalis* Thus bacteria most commonly seen in acute otitis media produced the greatest Cl_q binding capacity

In acute purulent otitis media disorders in the complement system have been demonstrated with decreased serum levels of Cl_q and the circulation of abnormal complexes comprising Cl subcomponents Clr Cls and $C\bar{l}r$ $C\bar{l}s$ $C\bar{l}$ inactivator (2) An interaction between certain bacteria and Cl_q may explain these complement abberations

It has been proposed that binding of Cl to bacteria could represent an early defence mechanism against microbial infections (9) However in acute otitis media it could also be speculated that Cl_q binding by bacteria contribute to Cl abberations which induce an impairment in C activation through the classical pathway which could lead to defective opsonization of the bacteria

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STRUCTURE AND FUNCTION OF HUMAN EFFUSION MACROPHAGES FROM PATIENTS WITH MALIGNANT AND BENIGN DISEASE

I Isolation Morphology Proliferation and Phagocytosis

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Hammerstrøm J Structure and function of human effusion macrophages from patients with malignant and benign disease 1 Isolation morphology proliferation and phagocytosis Acta path microbiol scand Sect C 88 191 200 1980

The inflammatory cell composition of pleural or ascitic effusion fluids from 13 patients with malignant disease and 8 patients with benign disease was analyzed The macrophage content in the effusions was $4\,1\times10^5 \pm 1\,3$ cells/ml (mean ± SEM) with large variation (range $0\,1 - 27\,9\times10^5$ cells/ml) among patients Major blood cell contamination was excluded by the finding of low red blood cell/nucleated cell ratios in the effusions Effusion macrophages were isolated by Ficoll/Isopaque centrifugation and plastic adherence Monolayers of > 90% α napthyl-esterase positive and/or phagocytic cells were produced in most experiments Adherent effusion cells incorporated low amounts of methyl ^{3}H thymidine (methyl ^{3}H TdR) Most cells in DNA synthesis were removed by trypsin indicating that they were not macrophages Lymphokine supernatants induced increased methyl ^{3}H TdR incorporation in adherent cells in 3 of 8 experiments and microscopic proliferation of phagocytic cells was evident in one experiment [illegible]
synthesis slightly Effusion macr[illegible]
blood monocytes The ability tc[illegible]
[illegible]
after 4–8 days *in vitro*

Key words Tumour lymphokines endotoxin *Corynebacterium parvum*

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The concept of mononuclear phagocytes being important effector cells in host resistance against neoplasia (13) is supported by numerous reports concerning the effects of *in vitro* manipulation of macrophages obtained from normal animals (see 13 for ref) Attempts to analyze the function of macrophages isolated directly from the tumour site are fraught with technical problems and much less is known about these presumably more relevant cells *In animal* models macrophages may be a prominent feature of tumour infiltrates (2 10 20 29 32 41 43) The [illegible]
lin[illegible]
to [illegible]
phage mediated tumour cell toxicity correlates positively with *in vivo* tumour regression (30) immuno-

genicity (25), and low metastasizing capacity (25, 41) in some, but not in all (12), systems

In man, mononuclear cell infiltration in cancer tissue is probably positively correlated with a favourable prognosis (5, 6, 24, 28) Functional analysis of tumour-infiltrating lymphocytes has often failed to demonstrate significant cytotoxic responses (35, 39) The infiltrating cells in solid human tumours often contain a significant fraction of macrophages (1, 22, 42) and macrophages may be more numerous in primary tumours than in metastases (1) Observations on the possible cytotoxic activity of human tumour-associated macrophages have been lacking until recently, but during the course of this work, two reports indicating that these cells may exert cytotoxic influence on tumour cells have appeared (26, 40)

Isolation of viable macrophages from solid human tumours requires prolonged exposure to enzymes (11, 40) that may modify macrophage function by, for instance, removal of membrane receptors (4) Enzyme exposure can, however, be circumvented by isolating macrophages from tumour-associated effusions

In this paper, I present data on the isolation *in vitro* culture, morphology and phagocytosis of human pleural or peritoneal effusion macrophages (PEM) from patients with malignant or benign disease In the following papers, the results of experiments analyzing the cytotoxic activity of these cells (17) and the lymphokine production in effusion lymphocytes (18) will be reported

MATERIALS AND METHODS

Human pleural or peritoneal effusion macrophages (PEM) Effusions were collected sterile on polyethylene plastic bags with heparin 10–20 IU/ml as anticoagulant None of the patients had received cytostatic treatment during the last 6 months Bacteriological tests made on all samples were negative An aliquot of the fluid was *centrifuged and rapidly air dried drop preparations* made from the pellet of unseparated cells The fluid was filtered through sterile gauze to remove gross debris and 150–200 ml layered in aliquots on cold (4 °C) Ficoll/Isopaque (Lymphoprep Nyegaard & Co Oslo) and centrifuged for 15 min at 800 G (14) The mononuclear cell layer was washed twice with Hanks balanced salt solution (HBSS) and the macrophage content evaluated in drop preparations stained with Giemsa Washed mononuclear effusion cells were suspended in RPMI 1640 (Gibco Bio Cult Glasgow) supplemented with 25% human AB Rh+ serum 0 1 mM l glutamine and [illegible]

Ø 16 mm) or on circular 14 mm glass coverslips [illegible]

the wells Adherence of macrophages was allowed for 90 min at 37 °C, 10% CO_2 The plates were shaken 3 times during this period to ensure contact of all macrophages with plastic The non adherent cells were then pipetted off, and the monolayers washed vigourously 6 times with jets of warm HBSS In some experiments the monolayers were exposed to 0 25% trypsin (Gibco) for 5 min after 3 washes followed by 3 additional washes with HBSS The macrophage content of the monolayers was assayed by phagocytosis of heat killed *Candida albicans* (19) or α naphthyl esterase staining (21) and cultures containing less than 90% phagocytic and/or diffusely esterase positive cells were not used for cytotoxicity studies (17) Cultures not used immediately for studies were supplied with 0 5 ml fresh HS M on day 1 4 and 8 of *in vitro* culture

Human monocytes were obtained from defibrinated venous blood by Ficoll/Isopaque centrifugation and plastic adherence as described in (14)

Cell counts Cells in suspension were counted on an automatic particle counter (Coulter Model Fn) Live cells in monolayer culture were counted visually in coverslip preparations mounted as a microchamber (14 44) by the aid of an eyegrid Differential counts were performed visually on May Grunwald Giemsa stained drop preparations of unseparated effusion cells counting at least 200 cells in each preparation Evaluation of the different cell types was made by standard criteria (23) Identification of PEM was checked against α naphthyl esterase stained preparations in cases of doubt The distinction between tumour cells and mesothelial cells was sometimes difficult and must be regarded as approximate in some of the malignant cases

Morphological studies were performed on coverslip preparations with a microchamber technique (14 44) Live cells were photographed with phase contrast optics (Leitz Laborlux) and automatic photographic equipment (Leitz Orthomat) on Agfapan 100 film

Phagocytosis of ¹²⁵I labelled Candida albicans was assayed as described in (19) Briefly the mononuclear phagocyte monolayers were incubated with 0 3 ml of fresh HS M with $3\ 33 \times 10^6$ heat killed ^{125}I labelled *Candida* particles per ml for 15 min Extracellular particles were removed by 3 washes with HBSS and the number of ingested particles per cell counted visually in coverslip preparations counting 30 phagocytic cells in each preparation *Digestion of ingested Candida* was assayed by adding 0 5 ml HS M incubating the cultures for 18 hours and then harvesting the cultures as described in (19) Radioactivity was determined separately in the cell free supernatant the detached cells and the adherent cell lysate by gamma radiation counting Digestion capacity was calculated as

$$100 \times \frac{\text{(cpm supernatant)}}{\text{(cpm adherent cells + cpm supernatant + cpm detached cells)}}$$

Cell detachment during the digestion period was calculated as

$$100 \times \frac{\text{(cpm detached cells)}}{\text{(cpm adherent cells + cpm supernatant + cpm detached cells)}}$$

Total ingested cpm was 33459 ± 7780 cpm (mean ± SEM n 4 PEM)

DNA synthesis in adherent cells was determined by adding 1 0 μCi methyl ^{3}H thymidine (methyl ^{3}H TdR sp act. 5 Ci/mM) per culture and harvesting and processing the cells for liquid scintillation counting after 5 hours of methyl ^{3}H TdR incorporation as described in (14)

Endotoxin (LPS), Corynebacterium parvum (Cp) and lymphokine (MAF) treatment LPS from *E coli* 026 B6 (Sigma) (19) 0 1 μg/ml killed Cp (*Propionobacterium acnidum* I CN 6966) (15) 5 μg/ml or Cp induced lymphokine supernatants containing monocyte activa ting factors (MAF) diluted 1 2 (15) was added to PEM cultures as described in (17)

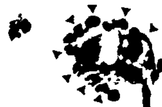


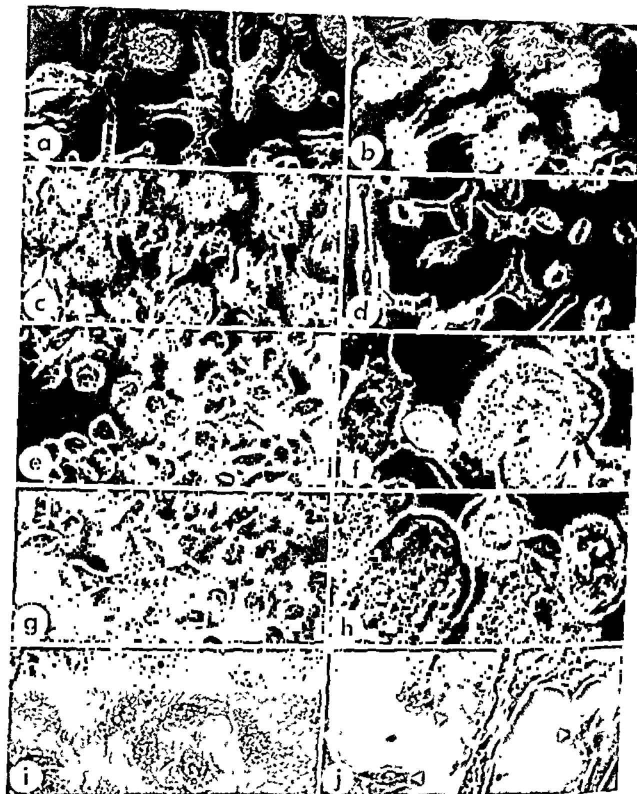
Fig 1 Three adenocarcinoma cells (large nuclei center) with attached inflammatory cells (Patient B) Cells morphologically identified as macrophages in the original preparation are indicated by arrows the rest are probably lymphocytes May-Grunwald-Giemsa-stained preparation of Ficoll/Isopaque-separated effusion cells × 400

TABLE 1 *Patients diagnosis composition of effusion cells and cell yield from the isolation procedure*

Patient	Diagnosis	Pleural or ascitic tumour[c]	% of nucleated effusion cells					RBC/leukocyte ratio	Cells × 10⁻⁴/l effusion[b]		PEM % in monolayer
			Tumour	Meso[d]	PMN[e]	Ly[f]	PEM		Mononuclear[g]	PEM	
	Malignant disease										
A	Adenocarcinoma[a]	+	5	1	1	79	14	1 5	1280	184	92 3
B	Adenocarcinoma[a]	+	10	2	10	41	37	2 5	1333	548	90 5
C	Adenocarcinoma (ovary)[a]	+	15	1	10	48	26	4 9	?000	577	9 1
D	Adenocarcinoma (colon)[a]	+	2	4	4	8	7?	6 5	350	?6?	98 7
E	Adenocarcinoma[a]	+	58	6	0	18	18	0 9	4000	720	91 4
F	Adenocarcinoma	+	6	0	9	70	15	1 8	118?	194	92 3
G	Squamous cell carcinoma (cervix uteri)[a]		5	10	?	5	78	? 8	3500	2785	94 8
H	Squamous cell carcinoma (bronchus)[a]		0	1	1	20	78	1 ?	70	56	96 4
I	Squamous cell carcinoma (bronchus)[a]		0	1	57	33	9	[illegible]	480	100	90 8
J	Mesothelioma (pleura)[a]	+	0	2	19	55	24	30 0	250	75	84 3
K	Mesothelioma (pleura)[a]		ND[h]	ND	ND	ND	ND	ND	175	53	75 7
L	Anaplastic small cell carcinoma (lung)[a]	+	1	?	7	70	20	0 2	2444	525	93 6
M	Breast carcinoma[a]	+	27	0	0	67	6	0 0	166	10	96 2
	Mean ± SEM		14 ± 6	3 ± 1	11 ± 6	41 ± 8	34 ± 9	4 5 ± 2 5	1345 ± 363	468 ± 203	91 5 ± 1 6
	Benign disease										
N	Pulmonary emboli[a]			0	0	98	?	3 0	?2?5	45	90 2
O	Pulmonary emboli[a]			12	1	39	48	1 3	142?	689	91 6
P	Cardiac failure (alcoholic cardiomyopathy)			11	4	69	16	? 5	4?8	71	93 1
Q	Cardiac failure ( )			0	71	2	19	2 0	255	231	93 0
R	Pleuritis (viral)			1	0	95	5	0 7	1185	59	71 0
S	Chronic pancreatitis			8	1	16	75	0 7	?750	946	97 4
T	Hepatic cirrhosis (alcoholic)[a]			24	0	7	69	0 0	500	345	97 1
U	Chronic hepatitis[a]			0	?	19	79	0 0	70	56	97 0
	Mean ± SEM			7 ± 3	10 ± 9	41 ± 14	39 ± 11	1 3 ± 0 4	916 ± 257	305 ± 1?0	91 3 ± 3 0

[a] Diagnosis verified ...
[b]
[d]
[e] Polymorphonuclear granulocytes
[f] Lymphocytes
[g] Including tumour cells and mesothelial cells
[h] Not done

All effusions except patient D were pleural effusions



RESULTS

Patient Material Composition of Effusion Cells and PEM Yield from Ficoll/Isopaque Separation

The differential cell counts in May Grunwald-Giemsa stained preparations of unseparated effusion cells the mononuclear cell yield and the macrophage yield from effusion fluid are given in Table I, along with the diagnosis Both the total cell content and the relative proportion of the different cell types found varied widely between patients, probably reflecting the heterogeneity of diseases and disease localization and stage The average macrophage yield from effusion fluid was $4\ 1 \times 10^5 \pm 1\ 3$ cells/ml (mean ± SEM), but with a wide range (Table I) The figures are not corrected for cell loss during

Fig 2 a PEM immediately after isolation (Patient C)

b PEM from the same effusion as in a (Patient C) 15 min after addition of *Candida albicans*

c PEM immediately after isolation (Patient L)

d PEM from the same effusion as in c (Patient L) after 18 hours of culture Note contraction of the cells and apparent reduction of phase-dense granules

e Blood monocytes immediately after isolation (Healthy control)

f Blood monocytes after 8 days of in vitro culture

g PEM with mainly monocyte like morphology (Patient Q) immediately after isolation

h PEM from the same effusion as in g (Patient Q) after 8 days of *in vitro* culture

i PEM with more differentiated macrophage morphology large well spread cells with numerous granules immediately after isolation (Patient S)

j PEM (arrows) from the same effusion as in i (Patient S) after 8 days of in vitro culture The other cells are proliferating mesothelial cells Live cells × 400

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separation so that the actual macrophage content in effusion fluid was probably somewhat higher

The red blood cell (RBC)/leucocyte ratio was low except in one case (Patient I) compared to the ratio of approximately 300 found in the peripheral blood of cancer patients (22) indicating that the presence of effusion leukocytes did not result from simple extravasation of blood into the serous cavity Macrophages and/or lymphocytes in contact with tumour cells even after vigorous pipetting and vortex mixing of the cell suspension were found in some effusions (Fig 1) No clear correlation between inflammatory cell content or type and diagnosis or disease progression could be found Data on survival were available for 10 patients with malignant disease In this group survival was 32 ± 35 (mean ± SD) days after collection of the fluid None had regressions that could not be attributed to conventional cytostatic or surgical therapy

Isolation of PEM

In prel [illegible]

[illegible] usually [illegible] removed by vigorous washing As mononuclear phagocyte spreading on plastic can occur in the absence of serum (8) effusion cells were plated in serum free RPMI 1640 in preliminary experiments This procedure led to increased sometimes uncontrollable clumping of effusion macrophages, and the number of adherent lymphocytes increased considerably while mesothelial and tumour cell contamination was reduced As effusion lymphocytes might be cytotoxic effector cells serum free plating was found unsuitable

Treatment of adherent cell cultures with 0 25% trypsin for 5 min efficiently removed both mesothelial and tumour cells leaving > 95% pure macrophage monolayers Trypsin did however, also remove 10–50% of the phagocytic cells in some experiments when the macrophages consisted of relatively small undifferentiated monocyte like cells (see Fig 2 g) [illegible]

[illegible] used in all experiments reported here and in (17) unless otherwise stated

[illegible] usually decreased steadily in unstimulated cultures (Fig 3) In about one third of the experiments proliferation of non phagocytic very motile cells of probable mesothelial origin resulted in cultures overgrown with this cell type after 1–2 weeks of culture (Fig 2 j) Tumour cell proliferation among the adherent cells was only seen in one experiment (Patient E) and these tumour cells developed into a cell line that was passaged for more than 5 months *in vitro* Interestingly the PEM isolated from this effusion was the only freshly isolated macrophage population that was found to enhance proliferation of the unrelated human tumour cell line NHIK 3025 (17)

Freshly isolated adherent effusion cells incorporated low amounts of methyl ^3H TdR (Table 2) and

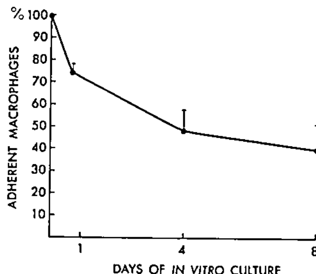


Fig 3 Survival of PEM *in vitro* Adherent macrophages were counted visually in 5 microscopic fields and expressed as per cent of the value found at culture start Mean ± SEM n = 6 at day 0 and day 1 n = 4 at day 4 and 8

TABLE 2 *Methyl ³H-thymidine Incorporation in Adherent Effusion Cells Effect of Incubation with LPS Cp and Cp induced Lymphokines (MAF), and Removal of Trypsin sensitive Adherent Cells*

	Treatment of adherent cells	Time of assay	cpm (mean ± SEM)	n
1	LPS[a]	Day 1	1315 ± 582	14
	HS M[b]		1775 ± 809	
2	Cp[c]	Day 2	2296 ± 1650	8
	HS M		2522 ± 1684	
3	MAF[d]	Day 2	2979 ± 1714	8
	HS M		2382 ± 1254	
4	MAF	Day 2	3022 ± 193	1[e]
	HS M		424 ± 3	
5	Wash[f] + LPS	Day 2	2214 ± 1036	7
	Wash + HS M		3273 ± 1687	
	Trypsin[g] + LPS		301 ± 75	
	Trypsin + HS M		439 ± 154	

[a] LPS 0 1 µg/ml added 18 hours before assay
[b] Medium (HS M) control
[c] CN 6966 5 µg/ml added on day 0 fresh HS M on day 1
[d] Cp induced lymphokines diluted 1 2 added on day 0 fresh HS M on day 1
[e] Patient Q
[f] Non adherent cells removed by six washes with HBSS
[g] Non adherent and trypsin sensitive cells removed by three washes with HBSS exposure to trypsin 0 25% for 5 min and three subsequent washes with HBSS

most of this incorporation was probably due to comtaminating mesothelial or tumour cells as it was reduced further by trypsin treatment (Table 2 line 5) No signs of proliferation of macrophages were seen morphologically in unstimulated cultures The spontaneous methyl ³H TdR incorporation in adherent cells from patients with malignant and benign disease was of similar magnitude The adherent cell response to LPS Cp and MAF was examined to see if the effects of these agents on macrophage mediated cytotoxicity (17) could be explained by proliferation of effector cells Treatment with LPS or Cp reduced methyl ³H TdR incorporation in adherent cells slightly (Table 2 line 1 and 2) while MAF induced an increase in methyl ³H TdR incorporation of adherent cells in three of eight experiments (Table 2 line 3) with a sevenfold increase in one (Table 2 line 4) In another experiment (Patient H) where PEM were cultured for 4 days in MAF clear evidence of proliferation was evident microscopically (Fig 4) with > 90% of the proliferating cells ingesting *Candida albicans*

Morphology and Differentiation of Effusion Macrophages

Blood monocytes when cultured under similar conditions differentiate from small cells with few or no granules to large macrophage like cells with abundant lysosomal granules during the first week of culture (Fig 2 e and f) Freshly isolated effusion macrophages were generally larger than monocytes spread more rapidly and more completely (Fig 2 a c i) and had considerable numbers of perinuclear granules (Fig 2 a c i) The morphological observations did not reveal any clear-cut structural differences between macrophages isolated from patients having malignant or benign disease Great variation of cell size spreading and granule content was present in both groups Effusions containing low numbers of mononuclear cells and high percentages of PEM often yielded monolayers of rather large well granulated macrophages Two main types of *in vitro* development were seen If the starting macrophage population were small and monocyte like (Fig 2 g) a considerable number of macrophages differentiated *in vitro* into large often multinucleated densely granulated cells (Fig 2 h) When the starting macrophages were large well granulated and well spread the cells contracted

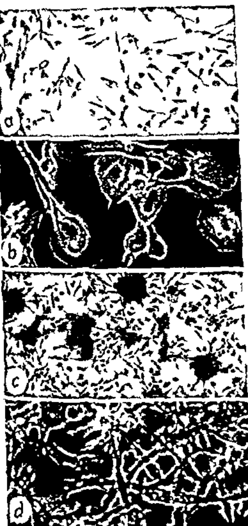


Fig 4 a PEM (Patient H) cultured for 4 days in HSM Live cells × 100

b High power (× 400) view of the same cells as in a with differentiated macrophage morphology

c PEM (Patient H) cultured for 4 days in lymphokine supernatants Proliferation with colony formation Live cells × 100

d High power (× 400) view of the same cells as in c Note membrane retraction reduction of lysosomal granules and vesicle formation

during the first 18 h of culture and the number of granules apparently diminished (Fig 2c and d) The cells could be induced to spread once more by addition of fresh serum but not to the original diameter If left undisturbed these cells remained in the contracted state dying gradually during day 4 to day 8 of culture without any further morphological signs of differentiation MAF exposure induced contraction of the cells and frequently vesicle like structures were seen usually to a lesser degree than in Fig 4 d

Phagocytosis and Digestion of Candida albicans

Freshly isolated effusion macrophages ingested more *Candida* particles per cell than autologous monocytes (Table 3 and Fig 2 b) The capacity to digest ingested fungi to soluble material released to the supernatant was larger and the effusion macrophages were far more adherent during the digestion period than monocytes (Table 3)

TABLE 3 *Phagocytosis and Digestion of ^{125}I labelled* Candida albicans

Mononuclear phagocytes	*Candida* particles per phagocytic cell	Digestion capacity %	Detached fraction %
PEM	13 3 ± 2 6 (6)	36 9 ± 6 9 (4)	10 4 ± 7 1 (4)
Monocytes*	3 7 ± 0 1 (6)	27 1 ± 1 0 (4)	56 9 ± 6 4 (4)

The mononuclear phagocytes were exposed to ^{125}I labeled *Candida* immediately after isolation (Day 0) The number of experiments is given in parantheses

* Autologous monocytes tested in parallel experiments

DISCUSSION

The availability of effusions removed from patients for diagnostic or therapeutic reasons offers a unique opportunity to study the structure and function of human macrophages »caught in the act« of exerting their actual *in vivo* function The heterogeneity of such non selected patients makes great caution necessary when one attempts to link *in vivo* condition to *in vitro* observations

The considerable contribution of macrophages to the inflammatory cell population resembles the situation found in solid human tumours (22 34 42) The reasons for the great variation in the proportion of macrophages which has also been noted previously (7) are obscure Exudate macrophages are probably mostly cells recruited from the circulating blood monocyte pool (37 38) If this holds true it seems evident from the data in Table 1 that mononuclear phagocytes in considerable numbers can be mobilized into malignant effusions containing tumour cells The presence of human tumour cell factors suppressing monocyte chemotaxis as reported in (31) does not seem sufficient to induce gross defects in mononuclear phagocyte mobilization into malignant effusions although the data are insufficient for strict quantitative comparison between the malignant and benign group Morphological evidence of inflammatory cell interaction with tumour cells in effusions (Fig 1) has been reported previously (33)

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The methyl ^{3}H-TdR incorporation and proliferation induced in PEM by MAF in some experiments suggest that local replication of macrophages in inflammatory sites may take place in man under certain conditions although studies in animal systems (37) indicate that the circulating blood monocyte is the main source of tissue macrophages Observations in our laboratory indicate that human blood monocytes may have considerable proliferative potential (36 *Hammerstrom & Eggen* unpublished)

Inflammatory macrophages isolated from patients with malignant or benign disease were not systematically different with regard to the properties studied The variation in cell structure spreading phagocytosis and proliferative response to lymphokines observed with cells from different patients probably reflects adaption of the mononuclear phagocytes to different inflammatory signals More homogenous groups of patients or longitudinal studies of individual patients are probably required to analyze mononuclear phagocyte response in malignant disease in more detail However the presence of considerable numbers of macrophages in human tumour associated effusions and the possibility of isolating these cells in reasonable purity by simple methods should encourage further investigations designed to clarify the role of human mononuclear phagocytes in natural resistance against cancer In the accompanying communication (17) the results of experiments examining the cytostatic and cytolytic activity of PEM is reported

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DISCUSSION

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STRUCTURE AND FUNCTION OF HUMAN EFFUSION MACROPHAGES FROM PATIENTS WITH MALIGNANT AND BENIGN DISEASE

2 *In Vitro Cytostatic and Cytolytic Effect on Human Tumour Cell Lines*

JENS HAMMERSTRØM

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Hammerstrøm J Structure and function of human effusion macrophages from patients with malignant and benign disease 2 *In vitro* cytostatic and cytolytic effect on human tumour cell lines Acta path microbiol scand Sect C 88 201–209 1980

Human effusion macrophages isolated from the pleural or ascitic effusions of 14 patients with malignant or benign disease usually inhibited methyl ^{3}H thymidine incorporation in an adherent human tumour cell line (NHIK 3025) when the macrophages were challenged with target cells immediately after isolation The cytostatic activity disappeared when the macrophages were cultured for 18 hours *in vitro* before target cell challenge The presence of endotoxin (LPS) or *Corynebacterium parvum* (Cp) during the macrophage target cell interaction induced a small enhancement of the macrophage mediated cytostatic activity Preincubation of macrophages with Cp or Cp-induced lymphokine supernatants for 2–18 hours before target cell challenge induced increased cytostatic activity in the macrophage cultures Adherent (NHIK 3025) or non adherent (K 562) human tumour cells prelabelled with methyl ^{3}H thymidine when added to freshly isolated macrophages were lysed in a slowly progressive manner The cytolytic activity to K 562 cells was enhanced by increasing macrophage density in the cultures and by incubating the macrophages for 2 hours with lymphokine supernatants before target cell challenge Morphological observations indicated that K 562 cells adhered to macrophage membranes with lysis proceeding extracellularly

Key words Cancer endotoxin *Corynebacterium parvum* lymphokines

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Studies on the function of human mononuclear phagocytes isolated directly from inflammatory sites are extremely few (see 9 for references) The paucity of studies actually demonstrating the ability of the human macrophage to effect cytotoxic responses to tumour cells is somewhat surprising considering that clinical trials with agents thought to augment macrophage mediated cytotoxicity to tumour cells have been carried out on quite a large [illegible] ount of data from animal systems (see 4 for references) provides a rationale for such trials

The cytotoxic activity of the human macrophage precursor, the blood monocyte, has been studied more extensively (5–8 10, 12, 14, 20, 21, 23, 24) Studies in our laboratory indicate that monocytes acquire cytostatic activity when differentiating *in vitro* (5 23) Lymphokines released from human lymphocytes stimulated with *Corynebacterium parvum* (Cp) or Bacillus Calmette-Guerin (BCG) can induce cytostatic (6, 10, 24) and cytolytic (7, 8)

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hile the incorporation in NHIK 3025 was unaffected (HS M 52167 ± 4234 cpm LPS 0.1 µg/ml 51968 ± 311 cpm n = 13)

Corynebacterium parvum (Cp) treatment Killed Cp (Propionobacterium avidum I CN 6966) (6) was suspended in HS M to twice the desired concentration and added to PEM or PEM/NHIK 3025 cultures as described for LPS The TI for cultures receiving Cp and target cells at the same time on day 0 was calculated with PEM and target cell controls without Cp (Table 2) The TI for cultures receiving Cp on day 0 and target cells on day 1 was calculated with PEM controls with Cp (PEM/HS M 2382 ± 1254 cpm PEM/Cp 5 µg/ml 1296 ± 1650 cpm n = 8) Killed Cp 5 µg/ml did not influence methyl ^{3}H TdR incorporation in NHIK 3025 cells cultured alone in separate control experiments

Lymphokine (MAF) treatment Lymphokine supernatants were produced by culturing peripheral blood lymphocytes from healthy individuals with killed Cp 2.5 µg/ml for 3 days as described in (10) Filtered (Millipore 0.45 µ) lymphokine supernatants were stored at +4 °C up to 2 weeks before use The medium was withdrawn from PEM monolayers and 0.5 ml lymphokine supernatants diluted 1 2 with fresh HS M added After the MAF exposure usually 18 hours the lymphokine supernatants were withdrawn and PEM monolayers washed once with HS M before addition of target cells as described under the cytostasis and cytolysis assay The TI was calculated with MAF treated PEM controls (PEM/HS M 2382 ± 1254 cpm PEM/MAF 2979 ± 1714 cpm n = 8)

Statistics Results given are mean of triplicate cultures ± SD for single experiments or mean ± SEM of n experiments P values were obtained by Wilcoxon s signed rank test for paired samples

RESULTS

Cytostatic Activity of Freshly Isolated PEM

Considerable cytostatic activity of PEM was found when target cells were added immediately after isolation (Table 1) in most experiments Three arbitrary levels of cytostatic activity might be discerned Three experiments with TI values negative or around 0 three experiments with very high TI values about 70 or higher, and the rest with values around 20 Two of the three patients with high TI values had a history of alcoholism No clear indication of correlation with effusion cell content or type of macrophage morphology was seen The cytostatic activity was strongly dependent on the effector/target cell ratio (E/T ratio) in one experiment where this was examined (Fig 1 curve indicated by HS M)

TABLE 1 *Cytostatic Activity of Effusion Macrophages to NHIK 3025 Cells*

Patient	Diagnosis	% Target cell inhibition (TI) (± SD)	
		Day 0[a]	*Day 1*[b]
	MALIGNANT		
A	Adenocarcinoma	+27.9 (1.6)	−2.7 (2.3)
B	Adenocarcinoma	+3.9 (3.0)	1.7 (1.6)
C	Adenocarcinoma (ovary)	+19.4 (0.5)	−2.1 (0.9)
D	Adenocarcinoma (colon)	+19.0 (3.2)	+7.5 (2.5)
E	Adenocarcinoma	−15.7 (4.4)	ND[c]
H	Squamous cell carcinoma (cervix uteri)	+17.7 (0.1)	ND
I	Squamous cell carcinoma (bronchus)	+71.4 (2.2)	−1.4 (4.1)
L	Anaplastic small cell carcinoma (lung)	+20.6 (1.9)	−2.1 (2.5)
M	Breast carcinoma	+2.6 (2.9)	−2.9 (3.2)
	Mean ± SEM	+18.5 ± 7.9	−0.8 ± 1.4
	BENIGN		
N	Pulmonary emboli	+23.2 (1.4)	−11.3 (0.8)
O	Pulmonary emboli	+19.1 (3.9)	−11.3 (3.6)
P	Cardiac failure (alcoholic cardiomyopaty)	+68.2 (1.1)	ND
T	Hepatic cirrhosis (alcoholic)	+94.2 (0.3)	ND
U	Chronic hepatitis	+20.7 (0.7)	−16.3 (2.7)
	Mean ± SEM	+45.1 ± 15.3	−13.0 ± 1.7

a Target cells (10^4/well) were added immediately after isolation of PEM
b Target cells (10^4/well) were added after 18 hours of *in vitro* culture of PEM
c Not done

activity in human monocytes A possible direct induction of monocyte mediated cytostasis by Cp has been reported (6) Endotoxin (LPS) enhances the expression of cytostatic and cytolytic activity by both normal and lymphokine activated monocytes (7) These studies indicate that human mononuclear phagocytes are capable of responding to activating or enhancing signals with increased cytotoxicity in much the same manner as murine macrophages Such responses have not however, been demonstrated in experiments with the cells supposed to actually effect *in vivo* mononuclear phagocyte mediated cytotoxicity, namely human macrophages isolated directly from the inflammatory site In the previous paper (9) I described the isolation and *in vitro* culture of effusion macrophages from patients most of which had malignant disease In this paper I present the results of experiments designed to examine the cytostatic and cytolytic activity of effusion macrophages from the same patients and the response of macrophage mediated cytostasis and cytolysis to lymphokines Cp and LPS

MATERIALS AND METHODS

Human pleural or peritoneal effusion macrophages (PEM) were isolated from heparinized effusion fluid by Ficoll Isopaque centrifugation and plastic adherence as described in (9) The PEM were cultured in 16 mm tissue culture wells (Costar 3524) in 0 5 ml RPMI 1640 (Gibco) supplemented with 25% pooled human AB Rh + serum 0 1 mM l glutamine and 40 µg gentamicin per ml (HS M) Only cultures with >90% macrophages as evaluated by phagocytosis of heat killed *Candida albicans* (5) or α napthyl esterase staining (11) were used for cytotoxicity studies unless otherwise stated

Target cells The adherent cell line NHIK 3025 originating from a human carcinoma *in situ* of the cervix (17) and the non adherent human myeloid leukemia cell line K 562 (13) were used as target cells Target cells were passaged in RPMI 1640 with 20% human A Rh + serum l glutamine and gentamicin

Assay for macrophage mediated cytostasis The medium (with lymphokine supernatants in some experiments) was withdrawn from PEM monolayers and 10^4 unlabelled NHIK 3025 cells prepared by trypsination (0 25% 3 min) added in 0 25 ml HS M Thereafter 0 25 ml HS M (with endotoxin (LPS) or *C parvum* in some experiments) was added Target cell DNA synthesis was assayed by adding 1 0 µCi methyl ^{3}H thymidine (methyl ^{3}H TdR sp act 5 Ci/mM) for the last 5 hours of a 18 hour coculture period of macrophages and target cells and harvesting and processing cultures for liquid scintillation counting as described in (5) The methyl ^{3}H TdR incorporation in PEM cultures without target cells and in target cells plated alone was determined simultaneously and experiments in which the methyl ^{3}H TdR incorporation in PEM cultures exceeded 10% of that found in target cells plated alone were excluded from the study (4 experiments usually owing to contamination with mesothelial cells) Macrophage mediated cytostasis was calculated as percentage target cell inhibition (TI)

$$100 - \frac{\text{cpm (NHIK 3025 + PEM)} - \text{cpm (PEM)}}{\text{cpm (NHIK 3025)}} \times 100$$

Methyl ^{3}H TdR incorporation in PEM on day 1 of culture was 1775 ± 809 cpm (n = 14) and on day 2 2106 ± 818 cpm (n = 11) Methyl ^{3}H TdR incorporation in NHIK 3025 cells plated alone was 57 830 ± 3458 cpm (n = 25)

Assay for macrophage mediated cytolysis Target cells in exponential growth were labelled with methyl ^{3}H TdR (0 5 µCi/ml for 24 hours or 1 0 µCi/ml for 8 hours) The medium (with lymphokine supernatants in some experiments) was withdrawn from PEM monolayers the monolayers washed once with HS M and 10^4 washed prelabelled target cells added in 0 25 ml fresh HS M followed by 0 25 ml HS M (with LPS in some experiments) The assay was terminated after incubation for 24 48 or 72 hours by addition of 0 5 ml HBSS to the well and aspiration of the supernatant which was centrifuged (400 G 10 min) The upper 0 5 ml of the supernatant was removed and radioactivity determined by liquid scintillation counting (23) Spontaneous release was determined in the upper 0 5 ml of the supernatant of target cells plated alone with or without LPS and maximal release in the supernatant of target cells receiving 0 5 ml 1% sodium dodecyl sulphate (SDS) instead of HBSS at termination of the assay Macrophage mediated cytolysis was calculated as % specific lysis (% SL)

$$100 \times \frac{\text{exp release} - \text{spon release}}{\text{max (SDS) release}}$$

Maximal (SDS) release was 13558 ± 2847 cpm (NHIK 3025 n = 4) and 6578 ± 1165 cpm (K 562 n = 4) which was >90% of total incorporated cpm Spontaneous release (% of SDS release) was NHIK/72 hours 23 2 ± 4 0% NHIK/LPS 0 1 µg/ml/72 hours 22 2 ± 4 2% K 562/48 hours 12 7 ± 8% (n = 4) Per cent SL in cultures with LPS was calculated with target [illegible] LPS concentration [illegible]

twice the desired concentration in HS [illegible] was removed from PEM monolayers 0 25 ml of HS M with 10^4 target cells was added and then followed by 0 25 ml of HS M with LPS Controls of target cells alone and PEM alone in HS M with and without LPS were included in each cytostasis experiment and the TI calculated with the controls exposed to the same concentration of LPS Methyl ^{3}H TdR incorporation in PEM was slightly reduced by LPS (HS M 1775 ± 809 cpm LPS 0 1 µg/ml 1315 ± 582 cpm n = 14 day 1)

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B	Adenocarcinoma	+ 3 9 (3 0)	– 1 7 (1 6)
C	Adenocarcinoma (ovary)	+19 4 (0 5)	– 2 1 (0 9)
D	Adenocarcinoma (colon)	+19 0 (3 2)	+ 7 5 (2 5)
E	Adenocarcinoma	–15 7 (4 4)	ND[c]
H	Squamous cell carcinoma (cervix uteri)	+17 7 (0 1)	ND
I	Squamous cell carcinoma (bronchus)	+71 4 (2 2)	– 1 4 (4 1)
L	Anaplastic small cell carcinoma (lung)	+20 6 (1 9)	– 2 1 (2 5)
M	Breast carcinoma	+ 2 6 (2 9)	– 2 9 (3 2)
	Mean ± SEM	+18 5 ± 7 9	– 0 8 ± 1 4
	BENIGN		
N	Pulmonary emboli	+23 2 (1 4)	–11 3 (0 8)
O	Pulmonary emboli	+19 1 (3 9)	–11 3 (3 6)
P	Cardiac failure (alcoholic cardiomyopaty)	+68 2 (1 1)	ND
T	Hepatic cirrhosis (alcoholic)	+94 2 (0 3)	ND
U	Chronic hepatitis	+20 7 (0 7)	–16 3 (2 7)
	Mean ± SEM	+45 1 ± 15 3	–13 0 ± 1 7

[a] Target cells (10⁴/well) were added immediately after isolation of PEM

[b] Target cells (10⁴/well) were added after 18 hours of *in vitro* culture of PEM

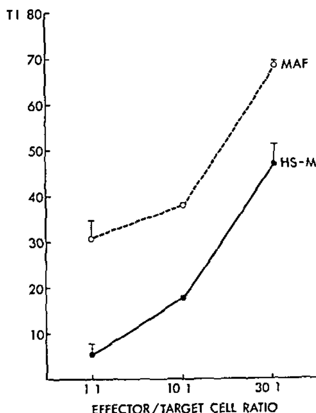


Fig 1 Influence of increasing macrophage density (effector/target cell ratio) and short (2 hours) preincubation with Cp-induced lymphokine supernatants (MAF) on the cytostatic activity of freshly isolated PEM, expressed as % target cell inhibition (TI) Target cells (NHIK 3025 cells, 10^4/well) were added 2 hours after isolation of PEM Mean ± SD of triplicate cultures n = 1 (Patient H)

Cytostatic Activity of PEM Cultured for 18 Hours in vitro

Overnight culture of PEM resulted in some cell loss (~25%) from the monolayers along with morphological changes reported in the previous paper (9) When target cells were added to PEM after 18 hours of culture, the cytostatic activity was reduced in all experiments (p = 0 001), the TI acquiring negative values (growth enhancement of target cells) in all experiments but one (Table 1)

Influence of LPS on the Cytostatic Activity of PEM

When LPS 0 1 μg/ml was present in the cocultures of freshly isolated PEM and NHIK 3025 cells, a slight to moderate enhancement of the cytostatic activity was found in most experiments (p = 0 007) (Table 2) In cultures of PEM challenged with target cells after overnight incubation, LPS produced a small enhancement in two experiments and an adverse effect in the third (Table 3)

Influence of Cp on the Cytostatic Activity of PEM

The presence of Cp 5 μg/ml in cocultures of freshly isolated PEM and target cells during effector/target cell interaction resulted in a small average enhancement of cytostasis (p = 0 029) (Table 2) When Cp was added to PEM cultures immediately after isolation and the cultures incubated for 18 hours before addition of target cells, some enhancement of cytostasis (p = 0 55) was found in 5 of 8 experiments (Table 3)

Influence of Preincubation with Lymphokine Supernatants on the Cytostatic Activity of PEM

Incubation with lymphokines released from human peripheral blood lymphocytes stimulated with Cp increase the cytostatic activity of human monocytes (10)

Freshly isolated PEM were exposed to lymphokines for 18 hours before the assay for cytostasis (Table 4) The enhancement of target cell growth usually found at this stage of PEM culture was reduced in all experiments (p = 0 008), changing to cytostasis in most experiments Four of the lymphokine supernatants used in this series of experiments were also assayed on blood monocytes cultured for 3 days *in vitro*, and in every instance induced a greater increase in TI in monocytes than in PEM However, differences in lymphokine storage time and mononuclear phagocyte culture time make a strict comparison of lymphokine responsiveness impossible The influence of increasing E/T ratio on the cytostatic activity of PEM exposed to lymphokines for only 2 hours after isolation was examined in one experiment (Fig 1)

Cytolytic Activity of PEM to Prelabelled NHIK 3025 Cells

When the PEM were challenged with methyl-^{3}H TdR-prelabelled NHIK 3025 cells immediately after isolation, a slowly progressing lysis of target cells was seen (Table 5) the main increase in lysis appearing from 48 to 72 hours of coculture LPS presence during PEM/target cell interaction did not induce reproducible effects in this small series of experiments

Cytolytic Activity of PEM to Prelabelled K-562 Cells

PEM cultures exposed to lymphokines or HS M for 2 hours after isolation were challenged with methyl-^{3}H-TdR-prelabelled K-562 cells at three different E/T ratios (Table 6) Consistent increase in target cell lysis was found after lymphokine activation at all E/T ratios The effect of increasing the effector cell density from E/T = *10 1* to E/T = 30 1 was dramatic in two of the four experiments

TABLE 2 *Effect of LPS and* C parvum *on the Cytostatic Activity of Freshly Isolated PEM to NHIK 3025 Cells*

Patient	% Target cell inhibition (TI) (± SD)				
	HS M	*LPS*	*TI change with LPS*	*CN 6966*	*TI change with CN 6966*
MALIGNANT					
A	+27 9 (1 6)	+33 0 (2 1)	+ 5 2	+31 1 (0 5)	+ 3 2
B	+ 3 9 (3 0)	+22 3 (3 8)	+18 4	− 0 6 (1 2)	− 4 5
C	+19 4 (0 5)	+20 9 (4 0)	+ 1 5	+23 5 (2 1)	+ 4 1
D	+19 0 (3 2)	+24 2 (3 5)	+ 5 2	+26 5 (1 3)	+ 7 5
E	−15 7 (4 4)	−22 0 (4 5)	− 6 3	−12 2 (0 9)	+ 3 5
I	+71 4 (2 2)	+84 7 (2 6)	+13 3	+70 3 (0 9)	− 1 1
L	+20 6 (1 9)	+49 4 (1 6)	+28 8	+44 6 (2 9)	+24 0
M	+ 2 6 (2 9)	+ 8 6 (5 6)	+ 6 0	ND[a]	−
Mean ± SEM	+18 6 ± 9 0	+27 6 ± 10 9	+ 9 0 ± 3 8	+26 2 ± 10 4	+ 5 2 ± 3 5
BENIGN					
N	+23 2 (1 4)	+41 1 (1 2)	+17 9	+32 0 (1 4)	+ 8 8
O	+19 1 (3 9)	+31 4 (4 3)	+12 3	+32 8 (0 2)	+13 7
P	+68 2 (1 1)	+67 4 (1 7)	− 0 8	+62 7 (1 6)	− 5 5
T	+94 2 (0 3)	+93 2 (0 1)	− 1 0	+93 3 (0 3)	− 0 9
U	+20 7 (0 6)	+23 3 (2 2)	+ 2 6	+25 1 (6 6)	+ 4 4
Mean ± SEM	+45 1 ± 15 3	+51 3 ± 12 8	+ 6 2 ± 3 8	+49 2 ± 12 8	+ 4 1 ± 3 4

Target cells (10^4/well) LPS 0 1 µg/ml and CN 6966 5 µg/ml were added immediately after isolation of PEM
a) Not done

human blood monocytes (8 23) K 562 cells frequently seemed to attach to PEM membranes without being ingested (Fig 2) with lysis apparently proceeding extracellularily

TABLE 3 *Effect of LPS and* C parvum *on the Cytostatic Activity of PEM Cultured for 18 Hours to NHIK 3025 Cells*

Patient	% Target cell inhibition (TI) ± (SD)				
	HS M	*LPS*[a]	*TI change with LPS*	*CN 6966*[b]	*TI change with CN 6966*
MALIGNANT					
A	− 2 7 (2 3)	ND	−	− 4 2 (2 4)	− 1 5
B	1 7 (1 6)	ND	−	+ 4 1 (2 5)	+ 5 8
C	− 2 1 (0 9)	ND	−	− 2 8 (1 2)	− 0 7
D	+ 7 5 (2 5)	ND	−	− 5 1 (2 2)	− 2 4
L	2 1 (2 5)	ND	−	+16 3 (3 7)	+18 4
M	− 2 9 (3 2)	+ 4 2 (3 6)	+ 7 1	ND	−
Mean ± SEM	0 7 ± 1 6	+ 4 2	+ 7 1	+ 3 7 ± 3 6	+ 3 9 ± 3 9
BENIGN					
N	−11 3 (0 8)	− 7 6 (2 5)	+ 3 7	+ 7 6 (0 8)	+18 9
O	−11 3 (3 6)	ND	−	− 7 6 (0 5)	+ 3 7
U	−16 3 (2 7)	−27 6 (5 6)	−11 3	+13 1 (1 8)	+29 4
Mean ± SEM	−13 0 ± 1 7	−17 6 ± 10 0	+ 3 8 ± 7 5	+ 4 4 ± 6 1	+17 3 ± 7 6

Target cells (10^4/well) were added 18 hours after isolation of PEM (Day 1)
a) LPS 0 1 µg/ml was added together with the target cells (Day 1)
b) CN 6966 5 µg/ml was added to PEM immediately after isolation (Day 0)

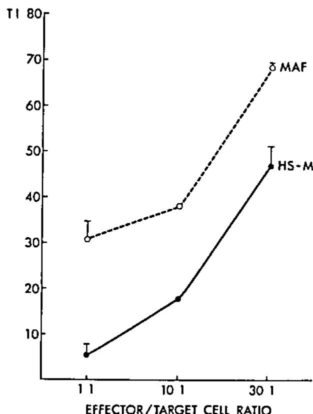


Fig 1 Influence of increasing macrophage density (effector/target cell ratio) and short (2 hours) preincubation with Cp induced lymphokine supernatants (MAF) on the cytostatic activity of freshly isolated PEM expressed as % target cell inhibition (TI) Target cells (NHIK 3025 cells 10^4/well) were added 2 hours after isolation of PEM Mean ± SD of triplicate cultures n = 1 (Patient H)

Cytostatic Activity of PEM Cultured for 18 Hours in vitro

Overnight culture of PEM resulted in some cell loss (~25%) from the monolayers along with morphological changes reported in the previous paper (9) When target cells were added to PEM after 18 hours of culture the cytostatic activity was reduced in all experiments (p = 0 001) the TI acquiring negative values (growth enhancement of target cells) in all experiments but one (Table 1)

Influence of LPS on the Cytostatic Activity of PEM

When LPS 0 1 μg/ml was present in the cocultures of freshly isolated PEM and NHIK 3025 cells a slight to moderate enhancement of the cytostatic activity was found in most experiments (p = 0 007) (Table 2) In cultures of PEM challenged with target cells after overnight incubation LPS produced a small enhancement in two experiments and an adverse effect in the third (Table 3)

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Freshly isolated PEM were exposed to lymphokines for 18 hours before the assay for cytostasis (Table 4) The enhancement of target cell growth usually found at this stage of PEM culture was reduced in all experiments (p = 0 008) changing to cytostasis in most experiments Four of the lymphokine supernatants used in this series of experiments were also assayed on blood monocytes cultured for 3 days *in vitro* and in every instance induced a greater increase in TI in monocytes than in PEM However differences in lymphokine storage time and mononuclear phagocyte culture time make a strict comparison of lymphokine responsiveness impossible The influence of increasing E/T ratio on the cytostatic activity of PEM exposed to lymphokines for only 2 hours after isolation was examined in one experiment (Fig 1)

Cytolytic Activity of PEM to Prelabelled NHIK 3025 Cells

When the PEM were challenged with methyl ^{3}H TdR prelabelled NHIK 3025 cells immediately after isolation a slowly progressing lysis of target cells was seen (Table 5) the main increase in lysis appearing from 48 to 72 hours of coculture LPS presence during PEM/target cell interaction did not induce reproducible effects in this small series of experiments

Cytolytic Activity of PEM to Prelabelled K-562 Cells

PEM cultures exposed to lymphokines or HS M for 2 hours after isolation were challenged with methyl ^{3}H TdR prelabelled K 562 cells at three different E/T ratios (Table 6) Consistent increase in target cell lysis was found after lymphokine activation at all E/T ratios The effect of increasing the effector cell density from E/T = 10 1 to E/T = 30 1 was dramatic in two of the four experiments

TABLE 6 *Cytolvt c Activity of Freshly Isolated PEM to K 562 Cells Prelabelled with Methyl ³H TdR Influence of E/T rat o and Short Preincubat on of PEM with Lymphokines*

Patient	% specific lysis (± SD)					
	1 1		10 1		30 1	
MALIGNANT	*HS M*	*MAF*[a]	*HS M*	*MAF*[a]	*HS M*	*MAF*[a]
H	3 4 (0 3)	6 7 (0 4)	6 8 (0 6)	10 3 (0 6)	34 9 (0 5)	54 8 (1 5)
K[b]	2 1 (0 4)	2 4 (0 9)	3 3 (1 0)	4 6 (1 0)	5 0 (1 0)	6 3 (0 4)
V[c]	3 9 (0)	5 1 (1 6)	8 2 (0 5)	9 5 (0 2)	60 7 (0 4)	68 3 (7 2)
BENIGN						
S	3 5 (0 7)	3 9 (0 5)	4 7 (1 1)	5 8 (0 9)	4 8 (0 3)	9 5 (0 6)
Mean ± SEM	3 2 ± 0 4	4 5 ± 0 9	5 8 ± 1 0	7 6 ± 1 4	26 4 ± 13 5	34 7 ± 15 7

Target cells prelabelled with methyl ³H TdR (10^4/well) were added 2 hours after isolation of PEM and isotope release determined after 48 hours of coculture

[a] Adherent effusion cells were preincubated with *C parvum* induced lymphokines d luted 1 2 in fresh HS-M for 2 hours before addition of target cells (Day 0)

[b] PEM purity 75 7% at culture start (mesothelial cell contamination)

[c] Diagnosis Bronchial carcinoma

tion by *Mantovani et al* (15) describes a more variable pattern of growth inhibition or stimulation of target cells cocultured with human macrophages isolated from ascitic ovarian carcinomas The



Fig 2 a Coculture of control PEM (HS-M) and methyl ³H TdR labelled K 562 cells (Patient H)

b Coculture of MAF-exposed PEM and K 562 cells from the same experiment

The rounded refractile K 562 cells adhere to the dark multipolar PEM Reduction of the number of K 562 cells is evident in the MAF-exposed culture Live cells photographed d rectly in the culture well on day 1 24 hours after target cell addition × 400

experimental conditions used by this group may account for some of this discrepancy Exposure to NH_4Cl buffer to lyse red cells has been shown to affect natural cytotoxic activity of some human mononuclear cells adversely (26) Human mononuclear phagocyte culture in medium supplemented with hetereologous serum affects cell survival and some cell functions adversely in our hands (23 *Hammerstrøm* unpublished data) Serum free pla ting of mononuclear effusion cells increases lym phocyte contamination of the monolayers (9) Finally the 48 h assay used by *Mantovani et al* is determining target cell growth at a stage of *in vitro* culture when all except one of the macrophage populations examined here had lost their initial cytostatic activity The cause of this initial disappea rance or reversal of mononuclear phagocyte cytotoxic activity which has also been described with human monocytes as effector cells (8) is not known The kinetics resembles the reversal of the cytotoxic activity seen with tumour associated macrophages isolated from regressing rat sarcomas (22) and with human monocytes activated with lymphokines *in vitro* (10) The cytostatic activity induced in human monocytes (5 23) or peritoneal macrophages (5) by *in vitro* differentiation for 4–8 days is stable and probably mediated by other mechanisms than the lymphokine induced cytotoxi city (8) The disappearance of the cytostatic activity mediated by freshly isolated PEM after one day of *in vitro* culture the apparent lack of correlation between cytostatic activity and differentiated mor phology in freshly isolated PEM (9) and the high

TABLE 4 *Effect of* C parvum-*Induced Lymphokines on the Cytostatic Activity of PEM Cultured for 18 Hours to NHIK 3025 Cells*

Patient	% Target cell inhibition (TI) (± SD)		
MALIGNANT	*HS-M*	*MAF*[a]	*TI change with MAF*
A	− 2 7 (2 3)	+ 6 7 (0 6)	+ 9 4
B	− 1 7 (1 6)	+ 4 4 (1 2)	+ 6 1
C	− 2 1 (0 9)	+ 7 6 (0 8)	+ 9 7
D	+ 7 5 (2 5)	+ 19 4 (1 5)	+ 11 9
L	− 2 1 (2 5)	+ 12 0 (1 2)	+ 14 1
Mean ± SEM	− 0 2 ± 1 9	+ 10 0 ± 2 6	+ 10 2 ± 1 3
BENIGN			
O	− 11 3 (3 6)	+ 22 6 (1 5)	+ 33 9
U	− 16 3 (2 7)	− 7 6 (1 0)	+ 8 7
Mean ± SEM	− 13 8 ± 2 5	+ 7 5 ± 15 1	+ 21 3 ± 12 6

Target cells were added 18 hours after isolation of PEM (Day 1)
[a]) PEM were exposed to *C parvum*-induced lymphokine supernatants diluted 1 2 for 18 hours before addition of target cells

DISCUSSION

The results presented here indicate that human macrophages isolated from an established *in vivo* inflammation may have considerable *in vitro* cytostatic influence on human tumour cells The mean cytostatic activity was higher in PEM from patients with benign disease, but the rather small and heterogenous groups do not allow conclusions to be drawn Work with animal systems has led to hypotheses of inefficient tumour cell killing by macrophages associated with progressing tumours (22, see also 4), and the data here justify further research to test such hypotheses in human systems

The only previous report on a similar phenome-

TABLE 5 *Cytolytic Activity of Freshly Isolated PEM to NHIK 3025 Cells Prelabelled with Methyl-³H-TdR*

Patient	% specific lysis (± SD)					
	24 h		48 h		72 h	
MALIGNANT	*HS M*	*LPS*[a]	*HS-M*	*LPS*[a]	*HS-M*	*LPS*[a]
D	2 9 (2 1)	0 9 (0 6)	3 0 (0 3)	4 3 (0 2)	14 9 (0 5)	9 4 (0 6)
G	1 0 (0 4)	2 0 (0 2)	4 6 (0 2)	5 6 (0 3)	10 5 (2 1)	14 6 (0 9)
Mean ± SEM	2 0 ± 0 9	1 5 ± 0 5	3 8 ± 0 8	5 0 ± 0 7	12 7 ± 2 2	12 0 ± 2 6
BENIGN						
O	1 6 (0 3)	3 0 (0 1)	4 0 (1 2)	6 8 (0 5)	17 2 (0 4)	20 2 (0 5)
R	3 7 (1 6)	−0 2 (0 1)	4 8 (2 0)	8 6 (1 4)	15 3 (1 5)	12 6 (2 2)
Mean ± SEM	2 7 ± 1 0	1 4 ± 1 6	4 4 ± 0 4	7 7 ± 0 9	16 3 ± 0 9	16 4 ± 3 8

Target cells prelabelled with methyl-³H TdR were added immediately after isolation of PEM and isotope release was determined after 24 48 or 72 hours of coculture
[a]) LPS 0 1 μg/ml (final concentration) was added together with the target cells

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cytolytic activity to K-562 cells observed in some experiments at high E/T ratios, may indicate that the cytotoxicity of freshly isolated PEM is more akin to lymphokine induced than to differentiation-induced monocyte cytotoxicity Assay of the cytostatic activity after 8 days *in vitro* of the PEM populations that underwent *in vitro* differentiation (9) was often complicated by the presence of rapidly proliferating mesothelial cells (9) In two experiments with little or no mesothelial cell contamination, a substantial increase in the calculated TI from day 2 to day 8 indicated that *in vitro* differentiating PEM again acquire tumour cell cytostatic activity after several days *in vitro,* in analogy with differentiating human monocytes or peritoneal macrophages (5)

Target cells may have varying susceptibility to macrophage-mediated cytostasis (15, 21) The choice of NHIK 3025 as target cells might explain the more uniform picture of cytostatic activity in these experiments, as opposed to the variable pattern of target cell growth found by *Mantovani et al* (15) with other target cells However, the K-562 line, which has been found by us to be susceptible to the cytostatic and cytolytic activity of human monocytes (8) and to PEM-mediated lysis in the experiments shown here, was found to be resistant to human macrophages (15) and monocytes (14) by *Mantovani et al* This may indicate that other factors possibly the noted differences in experimental technique, are responsible for the partly discrepant results

The influence of the E/T ratio on the cytostatic and cytolytic activity is in general agreement with *Mantovani et al* (15) However, macrophage density, rather than the relative number of target cells, seems to be the most important culture variable influencing the cytolytic activity both in animal (22) and human (8) *in vitro* systems This concept seems to be compatible with the experiments presented here It should be pointed out that the E/T ratios and especially the effector cell densities used in this study are lower than those regularly used for the demonstration of lymphocyte-mediated cytotoxicity (26)

Macrophage cytotoxicity has been shown to be associated with soluble substances released from macrophages in animal systems (1 2, 3) Furthermore, cold thymidine secreted by animal macrophages may interfere with cytostasis assays (18) PEM supernatants were not assayed for such activity in the experiments presented here The lack of evidence for thymidine like inhibitors in human monocyte (5 6 7 8 10, 14) and macrophage (15) supernatants the cytolytic effect observed here, and the results of preliminary experiments with PEM supernatants, suggest that cold thymidine secretion is an unlikely mechanism for the observed effects of PEM on target cell methyl-^{3}H-TdR incorporation

LPS can enhance the *in vitro* cytostatic and cytolytic response of differentiated and lymphokine-activated human monocytes (7) The *in vitro* response of PEM was generally low, and no indication of the presence of a noncytotoxic, LPS responsive stage of macrophage activation as described in a rat system (22) can be inferred from the data

Neither does Cp addition induce any dramatic increase in PEM cytostasis with the protocol used here Whether the observed increase in TI is caused by lymphokine-mediated or direct effects of Cp (6) on macrophage cytostatic activity cannot be ascertained from the present data, but in either case the effect is weak

Preincubation with Cp induced lymphokines increased PEM cytostatic activity, but again the level of activation was lower than that observed with normal human blood monocytes cultured for 3 days *in vitro* (6, 10) As the lymphokine and LPS responsiveness on normal human monocytes seem to decrease with advanced *in vitro* differentiation (7 8), one may suggest that PEM, isolated at a more advanced stage of *in vivo* differentiation than monocytes would behave more like *in vitro* differentiated monocytes Data supporting a similar hypothesis have been obtained in animal systems (19) A second series of experiments comparing the lymphokine responsiveness of PEM and monocytes from the same patient in terms of cytolytic activity is in progress

The limited data from the experiments with prelabelled target cells indicate that human macrophages can lyse human tumour cells, in agreement with (25) The kinetics of lysis the dependency on the macrophage density (E/T ratio), the activation by lymphokines and the probable extracellular progression of lysis are in line with previous data obtained with human monocytes cultured *in vitro* (7 8) The low levels of K-562 lysis during the first 24 hours make the contribution of natural killer (NK) cells unlikely, as NK-mediated lysis proceeds rapidly (26)

Caution is advisable when interpreting data such as these Clearly much work remains before a convincing link can be established between *in vitro* cytotoxic responses of human macrophages and the processes actually operating in a human malignant tumour

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IN VITRO RESPONSE TO *CORYNEBACTERIUM PARVUM* OF HUMAN EFFUSION LYMPHOCYTES ISOLATED FROM PATIENTS WITH MALIGNANT AND BENIGN DISEASE

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Hammerstrøm J *In vitro* response to *Corynebacterium parvum* of human effusion lymphocytes isolated from patients with malignant and benign disease Acta path microbiol scand Sect. C, *88* 211–218, 1980

Non adherent effusion cells (EC) mostly lymphocytes were isolated from the pleural effusions of 8 patients with malignant and 7 patients with benign disease *Corynebacterium parvum* (Cp) induced increased methyl ^{3}H thymidine (methyl ^{3}H TdR) incorporation in EC cultures but the response was lower than that usually found with autologous or allogeneic normal human blood lymphocytes Experiments with highly purified effusion lymphocytes indicated that the response to Cp was influenced by the presence of adherent cells probably macrophages Normal human monocytes incubated *in vitro* with supernatants of unstimulated EC cultures expressed slightly increased ability to suppress methyl ^{3}H TdR incorporation in a human tumour cell line Supernatants of Cp stimulated EC induced a further increase in monocyte mediated cytostatic activity Cell free effusion fluid from 8 patients were largely inactive when tested for induction of monocyte mediated cytostatic activity in the same system and the effusion fluid reduced monocyte methyl ^{3}H TdR incorporation *in vitro* Thus Cp seems to be able to induce DNA synthesis and release of mononuclear phagocyte activating lymphokines in human effusion lymphocytes

Key words Lymphokines cancer monocytes cytostasis proliferation

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Received 31 xii 79 Accepted 8 iv 80

Trials of non specific active immunotherapy of human cancer by local injection of Bacillus Calmette-Guerin (BCG) or *Corynebacterium parvum* (Cp) into the pleural or peritoneal cavity have indicated beneficial effects (24 26) also in randomized trials (18) One of the possible mechanisms may be local stimulation of lymphokine production with resultant activation of macrophages with anti-tumour cytotoxic activity (15) Interpleurally injected Cp is mainly distributed locally in mice (19) and BCG or Cp [illegible]

Cp with *in vitro* proliferation (6, 13, 16, 20) and lymphokine release (9, 10, 13, 16, 20) The lymphokines released can activate human blood monocytes (9, 10 13) and human effusion macrophages (12) to increased tumour cell cytotoxicity *in vitro* However, the functional response of human lymphocytes present *in situ* in the serous cavity inflammatory fluid (4, 5, 11) to Cp has not been described In this study, the ability of non-adherent human mononuclear cells isolated from malignant or benign effusions to respond *in vitro* to Cp with proliferation and lymphokine production was examined In addition, the results of experiments designed to detect monocyte activating lymphokine activity in cell free effusion fluid are reported

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IN VITRO RESPONSE TO CORYNEBACTERIUM PARVUM OF HUMAN EFFUSION LYMPHOCYTES ISOLATED FROM PATIENTS WITH MALIGNANT AND BENIGN DISEASE

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Trials of non specific active immunotherapy of human cancer by local injection of Bacillus Calmette-Guerin (BCG) or *Corynebacterium parvum* (Cp) into the pleural or peritoneal cavity have indicated beneficial effects (24 26) also in randomized trials (18) One of the possible mechanisms may be local stimulation of lymphokine production with resultant activation of macrophages with anti

Cp with *in vitro* proliferation (6 13 16 20) and lymphokine release (9 10 13 16 20) The lymphokines released can activate human blood monocytes (9 10 13) and human effusion macrophages (12) to increased tumour cell cytotoxicity *in vitro* However the functional response of human lymphocytes present *in situ* in the serous cavity inflammatory fluid (4 5 11)

benign effusions to respond *in vitro* to Cp with proliferation and lymphokine production was examined In addition the results of experiments designed to detect monocyte activating lymphokine activity in cell free effusion fluid are reported

MATERIALS AND METHODS

Human non-adherent effusion mononuclear cells (EC) were separated from 15 effusion fluids as described in (11) The cellular composition and mononuclear cell yield from 11 of the effusions have been described in (11) Mononuclear cells obtained by Ficoll/Isopaque centrifugation were washed twice in Hanks' balanced salt solution (HBSS), and suspended in RPMI 1640 (Gibco, Bio Cult, Glasgow) supplemented with 25% pooled human AB Rh + serum, 0 1 mM 1-glutamine and 40 μg gentamicin per ml (HS-M) The mononuclear cells were depleted of adherent cells (macrophages, mesothelial cells, and some tumour cells) by incubation on plastic for 90 min as described in (11) In some experiments, the EC obtained after incubation on plastic were depleted further of adherent cells by nylon wool column passage as described by *Greaves et al* (7), using a 5 ml column with 600 mg nylon fiber and RPMI 1640 with 10% human AB Rh + serum as absorption and elution medium Differential counts were performed visually on May-Grunwald-Giemsa-stained (MGG) and α naphtyl-esterase stained (ANAE) (14) drop preparations, as described in (11)

Human blood monocytes and lymphocytes from healthy volunteers were isolated from defibrinated venous blood by Ficoll/Isopaque centrifugation and plastic adherence of monocytes (8, 13) Blood lymphocytes from patients were isolated from heparinized venous blood samples drawn immediately before or after collection of the effusion fluid

DNA-synthesis in EC and blood lymphocytes was determined by culturing 10^6 cells in 1 ml HS-M in round bottomed 16 × 100 mm glass tubes as described in (13)

In some experiments where limited amounts of effusion cells were available 5×10^4 cells in 0 2 ml HS-M were cultured in the round bottomed wells of 96-well microplates (Sterilin Microtiter) *Corynebacterium parvum* (CN 6966) (9) 2 5 μg/ml (final conc) was added to some cultures at culture start In some experiments bacillus Calmette Guérin (BCG) (Statens Seruminstitut Denmark) or phytohemagglutinin (PHA) (Gibco) in optimal doses (9 22) was used for lymphocyte stimulation Two μCi methyl ^{3}H thymidine (methyl ^{3}H TdR) (sp act 5 Ci/mM) (1 ml-cultures) or 0 5 μCi methyl ^{3}H-TdR (0 2 ml cultures) was added to the cultures on day 5 of *in vitro* culture and the cultures were harvested after 5 hours of methyl ^{3}H-TdR incorporation with a Titertek cell harvester (22) The stimulation index (SI) was calculated as

$$\frac{\text{mean cpm (cells with Cp)}}{\text{mean cpm (unstimulated cells)}}$$

EC supernatants were produced by culturing 5×10^6 plastic adherence-purified EC (10^6 cells/ml in HS M) in 24 × 93 mm round bottomed glass tubes with or without Cp 2 5 μg/ml The EC supernatants were harvested by centrifugation (500 G, 10 min), and Millipore (0 45 μ) filtration after 3 days of EC culture The supernatants were stored at 4 °C for up to two weeks before use

Cell-free effusion fluid was obtained by centrifugation (500 G, 10 min) The fluid was stored at −20 °C for up to three months All effusion fluids were filtered before use (Millipore, 0 45 μ)

Assay for human monocyte-mediated cytostasis Monocyte monolayers cultured for 3 days in vitro (> 95% monocytes (9)) were incubated for 24 hours with EC supernatants diluted 1 2 with fresh HS-M, or cell-free effusion fluid The monolayers were then washed once, and the assay was performed with the human tumour cell line NHIK 3025 as target cells, as described in (13) The results are expressed as cytostatic index (CI)

$$100 - \frac{\text{cpm (treated monocytes + NHIK 3025)}}{\text{cpm (untreated monocytes + NHIK 3025)}} \times 100$$

The cytostatic activity of untreated monocytes (cultured in HS-M) is given for each series of experiments as per cent target cell inhibition

$$100 - \frac{\text{cpm (untreated monocytes + NHIK 3025)}}{\text{cpm (NHIK 3025)}} \times 100$$

Target cell cpm in the absence of monocytes was 62039 ± 433 cpm (mean ± SEM, n = 14)

Statistics Results are given as mean ± SD of triplicate cultures in single experiments or as mean ± SEM of n separate experiments P values were obtained by Wilcoxon s signed rank test for paired samples

RESULTS

Methyl-^{3}H-TdR Incorporation in EC and Blood Lymphocytes

The EC obtained after plastic adherence of mononuclear effusion cells contained low numbers of mesothelial cells, macrophages and, occasionally, tumour cells (Table 1) The rather high spontaneous methyl-^{3}H-TdR incorporation in the EC (Table 2) seemed to be related to the presence of such non-lymphoid cells, as nylon wool column passage of the plastic adherence-purified EC reduced non-lymphoid cell contamination to less than 1% (Table 1), simultaneously reducing the spontaneous methyl-^{3}H-TdR incorporation (Table 3) When adherent effusion cells (AEC, > 90% macrophages) were added back to nylon wool column-purified effusion lymphocytes in one experiment (Patient Y, Table 3), the spontaneous incorporation increased again Isolated mesothelial cells, but not isolated macrophages, proliferated spontaneously (11)

Plastic adherence-purified EC responded to Cp with increased methyl-^{3}H-TdR incorporation, but with a lower response than normal blood lympho-

TABLE 1 *Composition of Non-adherent Effusion and Blood Mononuclear Cells Purified by Plastic Adherence and Nylon Wool Column Passage*

Patient	Diagnosis	Cell source[a]	Cell treatment[b]	%[c]			
				Ly	Mp	Tumour	Meso
A	Adenocarcinoma	E	P	76 1	3 6	20 3	0 0
B	Adenocarcinoma	E	P	ND	ND	ND	ND
C	Adenocarcinoma	E	P	88 2	0 5	11 3	0 0
G	Squamous cell carc	E	P	90 7	0 3	7 8	1 2
H	Squamous cell carc	E	P	ND	ND	0 0[d]	ND
L	Anaplastic carc	E	P	97 2	1 5	1 0	0 3
Z	Breast carcinoma	E	P	89 2	6 6	0 0	4 1
		E	P + N	99 2	0 5	0 0	0 3
		B	P	89 7	10 3	–	–
		B	P + N	99 8	0 2	–	–
Y	Unclassified carc	E	P	93 5	3 7	2 8	0 0
		E	P + N	99 6	0 0	0 4	0 0
		B	P	93 8	6 2	–	–
		B	P + N	99 9	0 1	–	–
N	Pulmonary emboli	E	P	ND	ND	–	ND
O	Pulmonary emboli	E	P	96 0	3 0	–	1 0
Q	Cardiac failure (?)	E	P	95 8	4 2	–	0 0
R	Pleuritis (viral ?)	E	P	99 5	0 3	–	0 2
W	Endocarditis	E	P	97 9	2 1	–	0 0
S	Chron pancreatitis	E	P + N	99 1	0 5	–	0 4
		B	P + N	99 3	0 7	–	–
X	Atelectasis	E	P + N	99 0	1 0	–	0
		B	P + N	99 2	0 8	–	–

a) Effusion (E) or blood (B)
b) Plastic adherence for 90 min (P) and nylon wool column passage (N)
c) Percent lymphocytes (Ly) mononuclear phagocytes (Mp) tumour cells (Tumour) or mesothelial cells (Meso)
Differential counts in MGG stained EC preparations after 3 days of culture In experiments involving nylon wool column purification the differential counts were made on MGG and ANAE-stained preparations made immediately after purification
ND – not done

cytes (Table 2) Isolated macrophages (11) and mesothelial cells (data not shown) did not increase their methyl ^{3}H TdR incorporation in response to Cp Nylon wool column purified EC and blood mononuclear cells (> 99% lymphocytes) obtained from two patients responded to Cp (Patient S Table 3) and BCG (Patient X data not shown) Effusion lymphocyte response was lower than the blood lymphocyte response in both these experiments In two other experiments (Patient Z and Y Table 3), the nylon wool column passage almost totally abrogated the effusion lymphocyte response to Cp although these cells could still respond to PHA However the PHA response of autologous blood lymphocytes identically treated was somewhat higher The effusion cell response to Cp was restored by adding back adherent effusion cells (> 90% macrophages) to the nylon wool column purified effusion lymphocytes in one experiment (Patient Y Table 3) indicating that the Cp response may be dependent on the presence of adherent cells probably macrophages in the culture

Effect of EC Supernatants on the Cytostatic Activity of Normal Human Monocytes

The supernatants of unstimulated or Cp-stimulated plastic adherence purified EC cultures were assayed for lymphokine activity by determining their ability to induce cytostatic activity in normal human monocytes (Table 4) Small to moderate increases in cytostatic activity compared to untreated monocytes were usually seen with unstimulated

TABLE 2 *Methyl-3H-TdR Incorporation in Plastic Adherence-purified EC in Response to* C parvum

Patient	Non stimulated	CN 6966	
MALIGNANT	*cpm (± SD)*	*cpm (± SD)*	*SI*[a]
A	19 096 (2025)	64 026 (7401)	3 4
B	16 696 (9632)	21 995 (814)	1 3
C	6 544 (878)	34 909 (9055)	5 3
G	12 897 (4757)	30 287 (11103)	2 4
H	19 178 (1149)	44 996 (7370)	2 4
L	799 (214)	861 (352)	1 1
Mean ± SEM	12 535 ± 3045	32 854 ± 8700	2 7 ± 0 6
BENIGN			
N	555 (32)	4 488 (70)	8 1
Q	1 234 (159)	3 370 (538)	2 7
R	338 (47)	2 982 (743)	8 8
W	8 531 (1965)	38 003 (6984)	4 5
Mean ± SEM	2 664 ± 1964	12 210 ± 8603	6 0 ± 1 5
NORMAL BLOOD LYMPHOCYTES			
Mean ± SEM (n = 6)	1 973 ± 713	53 279 ± 6138	42 3 ± 9 9

a) Stimulation index

TABLE 3 *Methyl-3H-TdR Incorporation in Nylon Wool Column purified Effusion and Blood Lymphocytes from the Same Individual in Response to* C parvum *and PHA*

Patient	Cell source	Cell treatment	Non stimulated	CN 6966		PHA	
			cpm (± SD)	*cpm (± SD)*	*SI*	*cpm (± SD)*	*SI*
S	E	P + N	4 352 (417)	15 601 (775)	3 6	ND	–
	B	P + N	1 534 (405)	79 140 (5885)	51 6	ND	–
Z[a]	E	P	7 149 (111)	10 133 (595)	1 4	ND	–
	B	P	328 (26)	9 151 (471)	27 9	ND	–
Z[a]	E	P + N	308 (26)	764 (48)	2 5	4 592 (409)	14 9
	B	P + N	97 (13)	4 372 (397)	45 1	6 666 (360)	68 7
Y[a]	E	P	2 669 (152)	6 994 (341)	2 6	12 428 (1034)	4 7
	B	P	80 (18)	2 992 (364)	37 4	11 872 (1144)	148 4
Y[a]	E	P + N	173 (16)	170 (71)	1 0	10 030 (761)	58 0
	B	P + N	91 (20)	1 135 (56)	12 5	16 527 (1536)	181 6
Y[a]	E	P + N + AEC[b] 0 2%	*626 (164)*	*1 241 (200)*	*2 0*	ND	–
	E	P + N + AEC 2 %	4 452 (396)	7 157 (191)	1 6	ND	–
	E	P + N + AEC 20 %	6 431 (183)	14 546 (1670)	2 3	ND	–

a) Microtiter (0 2 ml) cultures

b) Adherent effusion cells (*AEC*) *purified* by plastic adherence (> 90 % macrophages) were removed by scraping with a *rubber policeman* washed in HS M, and added back to autologous column purified lymphocytes to give the indicated percentage of AEC in the culture

Other abbreviations as in Table 1 and 2

TABLE 4 *Cytostatic Activity Induced in Normal Monocytes by EC Supernatants*

EC supernatants from patient	Cytostatic Index (CI) (± SD)		
MALIGNANT	*Non stimulated EC supernatant*	*Cp stimulated EC supernatant*	*CI change with Cp*
A	+ 4 2 (3 6)	+ 21 4 (0 2)	+ 17 2
B	+ 13 4 (1 7)	+ 23 9 (1 3)	+ 10 5
C	+ 13 0 (1 6)	+ 28 3 (2 7)	+ 15 3
H	+ 0 4 (0 4)	+ 7 4 (1 5)	+ 7 0
L	+ 10 6 (0 2)	+ 17 3 (1 1)	+ 6 7
Mean ± SEM	+ 8 3 ± 2 6	+ 19 7 ± 3 5	+ 11 3 ± 2 1
BENIGN			
N	+ 0 2 (4 8)	+ 1 8 (4 5)	+ 1 6
O	+ 14 3 (0 1)	+ 24 3 (0 4)	+ 10 0
Q	+ 21 1 (1 9)	+ 19 5 (2 9)	− 1 6
W	− 3 8 (2 5)	+ 22 8 (1 9)	+ 26 6
Mean ± SEM	+ 8 0 ± 5 9	+ 17 1 ± 5 2	+ 9 2 ± 6 3

Normal human monocytes were incubated with EC supernatants diluted 1 2 from day 3 to day 4 of monocyte culture Target cells (NHIK 3025) in fresh HS M were added on day 4 Each EC supernatant was tested in one experiment in triplicate Percent target cell inhibition of untreated monocytes was + 4 5 ± 8 7 (mean ± SEM n = 9)

EC supernatants Cp addition to the EC cultures resulted in increased levels of monocyte activating activity in seven experiments and virtually no change in two

Effect of Cell free Effusion Fluid on Normal Human Monocytes

The possib l ty that cell f[illegible]

[illegible]

otocol similar to that used for EC supernatants wo of the fluids from patients with benign disease duced a small but significant enhancement in onocyte mediated cytostatic activity the rest being active (Table 5) To check for unstable monocyte tivating activity monocytes were exposed to [illegible]

[illegible]

owever only small effects of similar magnitude as ose shown in Table 5 were observed (data not own)

The effects of the effusion fluids on normal onocyte methyl ³H TdR incorporation were exa ined after 1–4 days of monocyte exposure to veral effusion fluid dilutions Suppression of onocyte methyl ³H TdR incorporation was found ith all protocols tested as exemplified in Table 6

DISCUSSION

This study indicates that non adherent cells, predominantly lymphocytes, from human malignant and benign effusions can respond to Cp *in vitro* with increased DNA synthesis and release of lymphokines capable of inducing enhanced cytostatic activity in human monocytes The effect [illegible]

Cp [illegible]

24

partly be due to such mechanisms The effusion cell respor[illegible]

isolate [illegible]

increa[illegible]

exposed to Cp Moreover, EC populations containing > 99 % lymphocytes gave reasonable proliferation responses in many experiments The data indicate that the effusion lymphocyte response to Cp PHA [illegible]

of auto[illegible]

tes Sı[illegible]

Low tr[illegible]

inflamı[illegible]

monstrated in lymphocytes isolated from rheumatoid arthritis synovial fluid (1) and solid human tumours (23), in spite of the high proportions of T-cells usually found (1, 21 23) The column-passaged EC were probably enriched in T-cells (7),

TABLE 5 *Cytostatic Activity Induced in Normal Monocytes by Cell-free Effusion Fluids*

Effusion fluid from patient	Cytostatic Index (CI) Mean ± SEM	p[a]
MALIGNANT		
E	−7 8 ± 8 6	n s
I	−2 7 ± 8 4	n s
J	+9 3 ± 11 1	n s
M	+5 9 ± 4 3	n s
Mean ± SEM	+1 2 ± 3 9	
BENIGN		
N	+9 0 ± 3 4	0 031
P	−6 5 ± 8 2	n s
T	−3 0 ± 6 6	n s
V	+6 7 ± 2 1	0 031
	+1 6 ± 3 7	
CONTROLS		
HBSS	−4 7 ± 2 5	n s
E tumour cell[b] supernatant	−2 2 ± 3 5	n s

Normal human monocytes were incubated from day 3 to day 4 of monocyte culture with cell-free effusion fluid, HBSS *or tumour cell supernatants* diluted 1 3 in HS-M Target cells (NHIK 3025) in fresh HS-M were added on day 4 Each effusion fluid was tested in five separate *experiments Percent target cell* inhibition of untreated monocytes was +5 0 ± 6 9 (mean ± SEM, n = 5)

a) Compared to untreated monocytes

b) *Cell-free 24-hour supernatant (HS-M) obtained from in vitro* passage no 17 of adenocarcinoma cells cultured from the effusion of patient E

TABLE 6 *Effect of Cell-free Effusion Fluid on Methyl-³H-TdR Incorporation in Normal Human Monocytes*

Effusion fluid from patient	Dilution	cpm (± SD)
A	1 3	533 (59)
	1 10	603 (36)
E	1 3	204 (28)
	1 10	627 (193)
None (HS-M)		1598 (46)

Normal human monocytes were incubated from day 3 to day 5 of *in vitro* culture with *cell-free effusion fluid* Methyl-³H-TdR incorporation was assayed on day 5 after a 5-hour pulse with 2 μCi methyl-³H-TdR/ml in fresh HS-M Mean of triplicate cultures

as < 85% of the lymphocytes exhibited the dot pattern of ANAE reactivity, which has been described as a T-cell marker (14) Considering that Cp is mitogenic for human T-lymphocytes (6), the data may be compatible with a reduced *in vitro* response to mitogens of effusion T-cells relative to circulating blood T-cells

Mononuclear phagocytes are required for antigen presentation to lymphocytes (22) and have been shown to regulate the proliferation in stimulated lymphocytes (22) The abrogation of the effusion *cell response to Cp by removal of adherent cells*, and the restoration of the response in one experiment by adding back such cells, may indicate that the [illegible] Cp [illegible]

[illegible]

inflammatory lymphocytes *is due* to different

functional abilities of inflammatory macrophages and blood monocytes remains to be examined

Adherent cells did also seem to be responsible for the high spontaneous methyl-³H-TdR incorporation in plastic adherence-purified EC Whether this resulted from the presence of spontaneously proliferating mesothelial or tumour cells, or from a stimulating effect of adherent cells on autologous effusion lymphocytes (2), cannot be decided from the present data

The experiments with EC *supernatants suggest* that effusion lymphocytes are capable of producing monocyte-activating lymphokines *in vitro* in response to Cp, *in the same system previously used to* demonstrate lymphokine release from human peripheral blood lymphocytes (9, 13) The induced cytostatic activity was somewhat lower than *that* usually observed with peripheral blood lymphocytes (9, 13) The cell yield in the experiments with column-purified lymphocytes was too low to allow direct comparison of the lymphokine production in blood and effusion lymphocytes

The failure to demonstrate monocyte-activating activity in the effusion fluid may be related to the experimental conditions Storage of effusion fluid may lead to inactivation of unstable factors In addition, the possible presence of inhibitory factors (17) or effusion fluid components influencing the *in vitro* monocyte culture conditions adversely should be excluded by fractionation of effusion fluids The data presented here are not sufficient to allow firm conclusions regarding the *in vivo* production of lymphokines by inflammatory cells Factors inducing *macrophage proliferation* have been described

by others in inflammatory exudates in rodents (25) and possibly also in man (3)

Further study of human cells recovered from inflammatory reactions seems to be required to test hypotheses based on *in vitro* observations or research on experimental animals

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AN IMMUNOELECTROPHORETIC ANALYSIS OF THE *STREP SANGUIS* AND ADULT HUMAN ORAL MUCOSA ANTIGEN EXTRACTS USED FOR IMMUNOLOGICAL INVESTIGATIONS OF RECURRENT APHTHOUS STOMATITIS

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Donatsky O An immunoelectrophoretic analysis of the *Strep sanguis* and adult human oral mucosa antigen extracts used for immunological investigations of recurrent aphthous stomatitis Acta path microb ol scand Sect C *88* 219-225 1980

Water soluble antigens of *Strep sanguis* strain 2A ATCC 10556 and adult human oral mucosa (AHOM) were analysed by four immunoelectrophoretic methods Crossed immunoelectrophoresis (CIE) using rabbit antibodies raised against the soluble antigens revealed 26 antigens in the standard *Strep sangu s* antigen extract and 16 antigens in the standard adult human oral mucosa extract All 26 Strep 2A antigen antibody precipitates observed in the CIE were identified as streptococcal components Eleven of the AHOM antigen antibody precipitates in the CIE were derived from human serum proteins and hemoglobin present in the crude AHOM Ag extract. Five of the latter precipitates poss bly were caused by oral mucosal antigens The AHOM Ag extract contained four bacterial contaminants None of them influenced the crossed immunoelectrophoretic AHOM antigen antibody reference pattern in the present study

Key words Stomatitis aphthous immunology electrophoresis

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Several immunologic investigations have shown .t cell mediated immunity (CMI) and humoral munity (HI) against oral mucosa antigens and eptococcal antigens are characteristic features *in* :ients with recurrent aphthous stomatitis (RAS) 6 7 8 9 10 11 12 13 14 15 16 22 23 24 · 26 27 28 29) These investigations however :re carried out using crude water-soluble antigen tracts of homogenized human oral mucosa and *'reptococcus sanguis* strain 2A ATCC 10556 The :esent study was undertaken in order to obtain a :tter identification of the various antigen compo- :nts For this purpose the antigens in some of the reviously used crude antigen extracts were analysed by immunoelectrophoretic methods Further it was attempted to obtain antigen antibody reference systems which later could be applied to screening of sera from patients with RAS for precipitating antibodies against adult human oral mucosa and *Streptococcus sanguis* strain 2A ATCC 10556

MATERIAL AND METHODS

Preparat on of ant gens Biopsy specimens from clinically normal unkeratinized oral mucosa were obtained from 100 clinically healthy persons with no signs or symptoms of RAS All biopsies were punch biopsies (diameter 5 mm) extending about 1 mm into the connective tissue The biopsy *specimens* were washed in sterile saline for

5–10 s snap frozen on dry ice and stored until preparation at −80 °C The oral mucosa extract was prepared as follows The biopsy specimens were cut into small pieces suspended in Hanks balanced salt solution (HBSS) and homogenized in an M S E rotating knife tissue homogenizer at 15000 rev/min for 7 minutes The homogenate was kept overnight at 4 °C and then centrifuged at 1000 × g for 20 minutes yielding 17 millilitres of supernatant with a protein concentration of 3 1 mg/ml (Lowry s method) The supernatant was stored in small aliquots at −20 °C Routine microbiologic examination of the supernatant was performed and no mycoplasms vira or fungi were isolated Bacteriologic examination however revealed α hemolytic oral streptococci *Actinomyces odontolyticus* *Staph aureus* and *E coli* These bacterial strains were stored at −80 °C and later prepared as outlined below

Five millilitres of the contaminated oral mucosa supernatant were sterile filtrated through an 0 22 µm Millipore® filter The effectiveness of filtration was established by a subsequent negative culture growth The sterile oral mucosa supernatant was stored in small aliquots at −20 °C

Strep sanguis strain 2A ATCC 10556 α hemolytic oral streptococci *Actinomyces odontolyticus* *Staph aureus* and *E coli* were used for the preparation of bacterial antigen extracts The *Strep sanguis* strain 2A was a subculture from a strain of *Strep sanguis* strain 2A that was originally isolated from recurrent aphthous ulceration and that appears to be related to or identical with *Strep sanguis* group H ATCC 10556 (4) The other bacterial strains were isolated from the present adult human oral mucosa extract These strains were identified according to usual bacteriologic principles in The Department of Clinical Bacteriology Institute of Medical Microbiology Rigshospitalet University of Copenhagen The five strains of bacteria were cultured separately on 5% horse blood agar supplemented by yeast extract The streptococcal strains were aerobically incubated at 35 °C for 3 days The strains of *Staph aureus* and *E coli* were similarly incubated for 30 hours The strain of *Actinomyces odontolyticus* was anaerobically cultured for 3 days in a glove box system as earlier described (21) The bacteria were scraped off the plates and washed three times in sterile 0 9% saline (12000 × g for 10 min) One gram wet weight of the different bacterial strains was suspended in 3 ml distilled H_2O and disintegrated by sonication performed at 20 [illegible]

staphylococci (gram positive bactaria) and 3 × 15 s for the strain of *E coli* (gram negative bacteria) The disintegrates were centrifuged at 48 200 × g for one hour at 4 °C The sonications resulted in the following colloid concentrations of the supernatants (measured by refractometry) *Strep sanguis* strain 2A 8 5 g/l α hemolytic oral streptococci 19 7 g/l *Actinomyces odontolyticus* 6 5 g/l *Staph aureus* 16 g/l and *E coli* 19 g/l The bacterial supernatants were stored in small aliquots at −20 °C

Preparation of rabbit antibodies The immunization of rabbits was carried out by Dakopatts Ltd Copenhagen Three rabbits were immunized with the extracts of bacterially contaminated adult human oral mucosa (AHOM Ag) sterile adult human oral mucosa (STERI AHOM Ag) and *Strep sanguis* strain 2A (Strep 2A Ag) respectively The injections were given intradermally with 100 µl of the antigen extract suspended in 100 µl of Freund s incomplete adjuvant at an interval of 14 days for the first four injections and of 4 weeks for the following four Bleeding was performed 10–12 days after the last of the first four injections and 10–12 days after each of the following four The rabbits were sacrificed 10–12 days after the last injections

Purification and concentration The rabbit immunoglobulin G and A fractions were purified and concentrated according to *Harboe & Ingild* (17) and stored at 4 °C The antibodies obtained from the different bleedings were immunoelectrophoretically analysed as described below Finally three standard reference pools of the antibodies (AHOM Ab STERI AHOM Ab and Strep 2A Ab) were obtained by mixing equal parts of antibodies from the different bleedings The protein concentrations (Lowry s method) of these three reference antibodies were AHOM Ab 9 0 g/l STERI AHOM Ab 10 5 g/l and Strep 2A Ab 9 0 g/l

Immunoelectrophoretic methods Immunoelectrophoretic analysis was performed using 1 Rocket immunoelectrophoresis (RI) 2 Crossed immunoelectrophoresis (CIE) 3 Crossed line immunoelectrophoresis (CLIE) with antigen in an intermediate gel and 4 Crossed immunoelectrophoresis with antibody in an intermediate gel (CIWIG) The equipment and procedures were as outlined by *Axelsen et al* (3) The gel was a one percent agarose gel (HSA Litex) in barbital buffer (pH 8 6 ionic strength 0 02) on 10 × 10 cm or 5 × 7 cm glass plates The first dimension electrophoreses were run at 12 °C applying 10 V per cm for one hour or 75 min respectively The second dimension electrophoreses and the rocket immunoelectrophoreses were run at 12 °C applying 2 5 V per cm for 20 h The gels were subsequently pressed dried stained (Coomassie brilliant blue) destained and dried The antibodies obtained at the different bleedings were screened by rocket immunoelectrophoresis with the respective antigens and antibodies in varying ratios Depending on the antibody responses as measured by rocket immunoelectrophoreses crossed immunoelectrophoreses were carried out using 10 µl of undiluted standard antigen extracts in the first dimensions and 8–15 µl of rabbit reference antibodies per cm² in the second dimensions gels The Strep 2A/Ra anti Strep 2A crossed immunoelectrophoretic patterns were checked for possible antibodies raised against the culture medium Rocket immunoelectrophoresis crossed immunoelectrophoresis and crossed line immunoelectrophoresis were performed with sonicated culture medium extract and Ra anti Strep 2A in varying antigen antibody ratios The AHOM/Ra anti AHOM crossed immunoelectrophoretic patterns were examined for possible precipitates derived from contaminating bacteria human serum proteins or

erythrocyte components in the AHOM extract used for immunization Rocket immunoelectrophoresis crossed immunoelectrophoresis and crossed line immuno electrophoresis were performed with the bacterial extracts standard human serum (Behring Institute) and erythrocyte membrane extract (EMB) (5) against the rabbit anti AHOM antibodies in varying antigen antibody ratios Crossed immunoelectrophoresis with antibody in an intermediate gel was performed with rabb t anti human whole serum protein (20 µl/cm²) (Dakopatts code no 100 SF) or rabbit anti EMB antibodies (20 µl/cm²) (Behring Institute and Dakopatts code no A104) in the intermediate gels The same analysis was performed with 10 µl of EMB extract in the first dimension run rabbit anti EMB (12 µl/cm²) in the second d mension gel and raised Ra anti AHOM (20 µl/cm²) in the intermediate gel The antigen extract and antibod es used for the EMB reference system were kindly provided by Dr *O J Bjerrum* (5) The rabbit pre mmunization sera were screened by crossed imm - n

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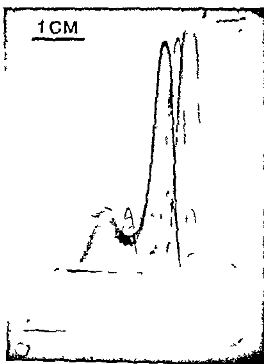
preimmunization serum per cm² in the second dimension gel

Absorpt on experiments The Ra anti AHOM antibodies raised were passed through CNBr activated Sepharose 4B columns (Pharmacia Fine Chemicals) coupled with standard human serum (Behring Institute) concentrated to the original volume with an »Immersible Molecular Separator Kit« (Millipore®) and absorbed with erythrocyte lysate (100 µl lysate 1 ml washed packed erythrocytes + 9 ml distilled H_2O) added to 5 ml of rabb t antibodies)

RESULTS

All the three standard antigen extracts (Strep 2A Ag AHOM Ag and Steri AHOM Ag) used for the rabbit immunizations induced similar immune responses The number of precipitates did not change from the first bleeding to the final bleeding However the antibody titres against the different antigens increased In the final study therefore the antibodies obtained from the different bleedings were mixed by equal parts resulting in three standard antibody pools (Strep 2A Ab AHOM Ab and STERI AHOM Ab)

The immunoelectrophoretic pattern of Strep 2A Ag and Strep 2A Ab is shown in Fig 1A Twenty six precipitation lines could be observed *The* precipitates were enumerated from the anode as indicated *in* Fig 1B Sonicated culture medium run against Strep 2A Ab with varying antigen antibody ratios did not reveal any precipitates Furthermore CLIE with sonicated culture medium in the intermediate gel did not change the Ag Ab reference pattern No precipitates were revealed if preimmu



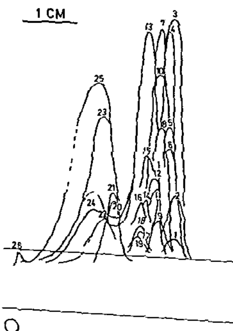
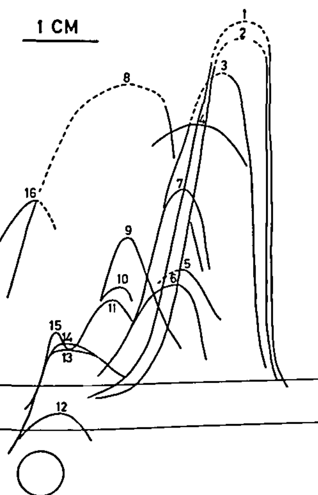


Fig 1 A Crossed immunoelectrophoresis with intermediate gel (CIWIG) of Strep 2A Ag (4 µl) against Strep 2A Ab (10 µl/cm²) Saline *in* the intermediate gel Twenty six precipitates were demonstrable
B Enumeration of the precip tates in Fig 1A



Fig 2 A Crossed immunoelectrophoresis with intermediate gel (CIWIG) of AHOM Ag (40 µl) against AHOM Ab (16 µl/cm²) Saline in the intermediate gel Sixteen precipitates were demonstrable
B Enumeration of the precipitates in Fig 2 A



nization serum was used in the second dimension gel of CIE together with Strep 2A Ag in the first dimension run

The immunoelectrophoretic pattern of AHOM Ag and AHOM Ab is shown in Fig 2A Sixteen precipitation lines could be observed The precipitates were enumerated from the anode as indicated in Fig 2B No precipitates were revealed if preimmunization serum was used in the second dimension gel of CIE together with AHOM Ag in the first dimension run

CLIE with the sonicated extracts of α hemolyt oral streptococci *Actinomyces odontolyticus Stapl aureus* or *E coli* in the intermediate gel did nc change the AHOM Ag Ab reference pattern

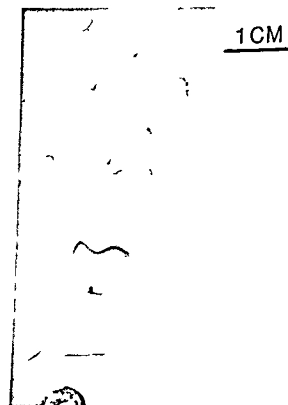


Fig 3 Crossed immunoelectrophoresis with intermediate gel (CIWIG) of AHOM Ag (40 µl) against AHOM Ab (32 µl/cm²) Prior to the electrophoretic analysis the AHOM Ab were passed through a CNBr activated Sepharose 4B column coupled with standard human serum Seven precipitates (no s 6 8 11 12 14 15 and 16) could still be seen

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Fig 4 Crossed immunoelectrophoresis with intermediate gel (CIWIG) of AHOM Ag (40 µl) against AHOM Ab (32 µl/cm²) Prior to the electrophoretic analysis the AHOM Ab were passed through a CNBr activated Sepharose 4B column coupled with standard human serum followed by absorption with erythrocyte lysate Five prec p tates (no s 6 11 12 14 and 15) could still be seen

CLIE and CIWIG with standard human serum (SHS) or rabbit anti human whole serum protein antibodies in the intermediate gel revealed that the reference precipitates no s 1 2 3 4 5 7 9 10 and 13 (Fig 2 B) were derived from human serum proteins (HS) and rabbit anti HS present in the AHOM Ag and the AHOM Ab respectively

Figure 3 shows the AHOM Ag/AHOM Ab immunoelectrophoretic pattern after the AHOM Ab were passed through the CNBr-column coupled with standard human serum The precipitates no s 1 2 3 4 5 7 9 10 and 13 have disappeared

The CLIE and CIWIG analysis with EMB and anti EMB antibodies revealed that precipitate no 8 (Fig 2 B) was derived from hemoglobin (Hb) and rabbit anti Hb present in the AHOM Ag and the AHOM Ab respectively Precipitate no 16 could not be identified in the present EMB reference system (5)

Figure 4 shows the AHOM Ag/AHOM Ab immunoelectrophoretic pattern after passage of the AHOM Ab through the CNBr-column followed by absorption with erythrocyte lysate The precipitates no s 1 2 3 4 5 7 8 9 10 13 and 16 have disappeared

The STERI AHOM Ag/STERI AHOM Ab immunization pattern was similar to the AHOM Ag/AHOM Ab pattern as far as number of precipitates identification of precipitates and increasing antibody titres were concerned

DISCUSSION

The results show that the methods for production of antisera and isolation of IgG and IgA according to *Harboe & Ingild* (17) are applicable to obtaining antibodies against crude water soluble Strep 2A AHOM and STERI AHOM antigens They furthermore show that various immunoelectrophoretic methods are applicable to establishing Strep 2A and AHOM Ag Ab systems as well as to identification of antigen components in crude standard Strep 2A and AHOM antigen extracts

With 26 different precipitates the Strep 2A Ag/Strep 2A Ab reference precipitation pattern obtained by crossed immunoelectrophoresis (CIE) certainly is complex However none of the precipitates apparently was derived from culture medium components or from antibodies raised owing to insufficient wash of the streptococci

Combined with a reference precipitin system crossed immunoelectrophoresis with an intermediate gel (CIWIG) has been found a powerful method for characterization of antibodies in mucocutaneous candidiasis *Pseudomonas aeruginosa* infections *Mycobacterium leprae* infections and *Actinomyces israelii* infections as well as for characterization of antibodies against *Bordetella pertussis* in human sera (1 2 18 19 20) In sera from patients with recurrent aphthous stomatitis (RAS) immunofluorescence studies have shown a significantly raised level of antibodies against *Strep sanguis* strain 2A (10 11 13) The present Strep 2A Ag/Strep 2A Ab reference system therefore seems promising for future characterization of antibodies against Strep 2A in sera from patients with RAS

The immunoelectrophoretic investigations revealed that the crude AHOM extract was contaminated with four strains of bacteria The α hemolytic streptococci and *Actinomyces odontolyticus* possibly are contaminants from the normal oral bacterial flora The *Staph aureus* and the *E coli* most likely are contaminants owing to non sterile procedures The counts of bacteria were small with no influence on the AHOM Ag/Ab reference system

However the immunoelectrophoretic analyses as well as the absorption experiments suggest that

most of the water-soluble antigen components of the crude AHOM extract are human serum proteins and hemoglobin The corresponding precipitins in the AHOM-Ab were absorbable resulting in five reference precipitates (no s 6, 11, 12 14 and 15, Fig 2B) These five precipitates seem to be derived from adult human oral mucosal antigen components and their corresponding rabbit antibodies

Experiments on the identity and specificity of the non absorbable AHOM-Ab are now in progress The present AHOM-Ag/Ab reference system seems promising for future characterization of oral mucosal autoantibodies and autoantigens in recurrent aphthous stomatitis

Finally, the results stress that before any further investigations of cell mediated immunity (CMI) in RAS are performed it is important to remove human serum proteins and hemoglobin from the human oral mucosal antigen extract These components probably have been present in all the human oral mucosal extracts previously used for CMI investigations in RAS (6 7, 9 10 11, 24, 27)

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Acta path microbiol scand Sect C 88 227-231 1980

GROUP B MENINGOCOCCAL OPSONINS IN SERUM MEASURED BY POLYMORPHONUCLEAR LEUKOCYTE CHEMILUMINESCENCE

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Lehmann V Solberg C O Group B meningococcal opsonins in serum measured by polymorphonuclear leukocyte chemiluminescence Acta path microbiol scand Sect C 88 227-231 1980

Serum opsonic activity against a strain of group B meningococci was measured by the initial increment in polymorphonuclear leukocyte chemiluminescence Chemiluminescence was markedly enhanced by serum and was dependent upon both thermostable and thermolabile factors It was higher in convalescent sera from patients with meningococcal disease than from other patients and normal controls Absorption of sera with the homologous strain substantially decreased the opsonic activity Convalescent serum from a patient with group C meningococcal pericarditis showed high opsonic activity towards a group B meningococcal strain A convalescent serum showed significantly higher activity towards its homologous group B strain than towards another group B meningococcal strain As measured by chemiluminescence properties besides group specificity are of importance in opsonization of meningococci The simplicity and precision of the chemiluminescence measurements seem to offer an improved tool for the study of meningococcal disease immunology

Key words Opsonins meningococci chemiluminescence

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Received 21 xi 79 Accepted 11 iii 80

Meningococci require specific antibody and heat labile serum factors in order to be fagocytosed and killed by polymorphonuclear leukocytes (PMN 6 7) Opsonization of the bacteria is therefore of vital importance for adequate host defence against meningococcal disease

Recently a sensitive technique has been developed which measures activation of PMN during phagocytosis (1) Following particle ingestion the microbicidal mechanism of the PMN is activated and excited molecular oxygen and carbonyl groups are generated Upon relaxation to the ground state these molecules emit photons resulting in a chemiluminescence (CL) which can be detected and quantitated in a liquid scintillation counter Recently this technique has been used for the measurement of streptococcal opsonins (4) and nonspecific opsonic activity (5)

The purpose of the present study was to examine whether the CL technique could be employed for the measurement of the opsonic activity of serum against meningococci

MATERIALS AND METHODS

Leukocyte preparation 10 parts freshly drawn heparinized (18 U/ml) whole blood were mixed with 3 parts dextran solution (60 mg/ml Pharmacia Sweden) and sedimented at room temperature After centrifugation of the supernatant fluid (500 *g* 5 min) and washing twice in phosphate buffered saline (PBS – 4500 ml distilled [illegible]

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[illegible] and the PMN concentration adjusted to 1 10^7 cells/ml Monocytes were present in amounts of 2–16 per cent

Bacteria Bacterial strains were kindly supplied by the Dept of Microbiology, The Gade Institute, University of Bergen Unless specified, the experiments were performed with a strain of *Neisseria meningitidis* group B (12809/79) isolated from a 45-year old woman with meningitis The original strain was kept freeze-dried and always used within 5 passages of the original isolation The bacteria were cultured overnight on heart infusion broth (Difco) agar plates (1 5 g/dl, D S T agar Oxoid) at 37 °C in the presence of CO_2 and moisture For phagocytosis experiments, plate scrapings were inoculated in heart infusion broth containing 0 1 g/dl of agar and incubated aerobically at 37 °C with vigorous shaking The bacteria were harvested in the log phase (2½–3 h) After centrifugation the bacterial pellet was washed in PBS and then resuspended in HBSS containing 0 5 g/dl of albumin, to an optical density of 1 00 (Beckman DB spectrophotometer, 620 nm, 10 mm light path) In this solution, the bacteria retained their viability for 2 h at room temperature Enumeration of viable bacteria was performed by spreading serially diluted samples on heart infusion broth agar plates An optical density of 1 00 corresponded to 3 8 ± 1 6 10^8 colony forming units/ml (mean ± 2 standard deviations)

Sera Homologous convalescent serum to strain 12809/79, drawn 3–8 weeks after the onset of disease, were used in most experiments In some instances (see text), other convalescent or normal sera (pooled human serum, PHS) were employed, and guinea pig serum was used as a source of complement The sera were kept at −70 °C, and when specified, inactivated by heating at 56 °C for 30 min prior to phagocytosis experiments For absorption studies, 1 ml of convalescent serum diluted 1 1 in PBS was incubated for 30 min at 4 °C with 2 10^9 homologous bacteria and regained by centrifugation prior to the measurement of opsonic activity Equally diluted controls were included

In one experiment portions of 1 2 10^8 bacteria were incubated for 30 min at 37 °C with 0 2 ml of either homologous convalescent serum heat inactivated serum with or without complement or complement alone After removing the supernatant fluid by centrifugation the bacterial pellets were washed in PBS resuspended in HBSS with albumin and examined by the CL procedure (vide infra) to determine the efficiency of preopsonization

Chemiluminescence procedure 0 5 ml bacterial suspension 0 5 ml PMN suspension 0 2 ml serum (when used) 0 2 ml complement (when used) and PBS to a final volume of 3 5 ml were preheated to 37 °C then mixed in Beckman Poly Q scintillation vials and kept at 37 °C Controls without bacteria serum complement or PMN were included The vials were kept in darkness and all procedures were carried out in shaded light CL was read as mean counts per minute (CPM) for periods of 30 s at 10 min intervals in a Beckman 100_c scintillation counter out of phase and with one photomultiplier disconnected To assess the opsonic activity of serum the CL counts from controls devoid of serum were subtracted at each time interval

Phagocytosis and bacterial killing To visualize phagocytosis during the CL procedure, 1 drop of reaction mixture was dispersed in 1 drop of albumin solution (5 g/dl) on a microscope slide, spread, dried fixed in methanol, stained with Giemsa solution and examined microscopically The concomitant killing of bacteria was determined by transferring 0 01 ml of the reaction mixture to 1 ml distilled water to lyse PMN (6) and then diluting further in HBSS with albumin to determine the total number of surviving meningococci

RESULTS

The addition of serum to a mixture of bacteria and PMN resulted in a marked increase in CL (Fig 1) Homologous convalescent serum induced a more rapid increase and a higher peak than PHS Controls devoid of serum showed low CL counts, increasing slowly with time Simultaneously a rapid and marked decrease in the number of viable bacteria occurred in the reaction mixture containing serum, while controls showed only moderate changes in bacterial viability (Fig 2) In the bacterial concentration range used, bactericidal activity of serum was not demonstrated Microscopic examination revealed that the addition of serum resulted in a marked ingestion of bacteria by PMN, while in the

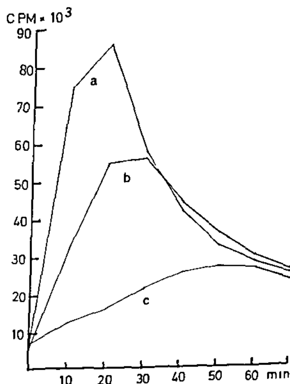


Fig 1 Chemiluminescence in reaction mixtures of polymorphonuclear leukocytes and meningococci in the presence of homologous convalescent serum (a) pooled human serum (b) and in the absence of serum (c)

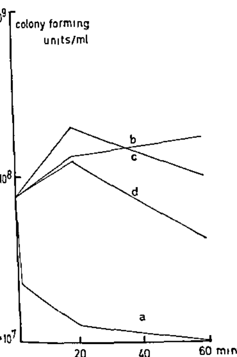


Fig 2 Total number of viable meningococci in a reaction mixture of polymorphonuclear leukocytes meningococci and serum (a) Controls without serum (b) without leukocytes (c) and bacteria alone (d)

absence of serum phagocytosis could not be demonstrated in 60 min (Fig 3)

The relationship between CL and the concentration of serum is shown in Fig 4 Peak values proved to be an unreliable expression of the CL activity because high activity yielded sharp peaks and the interval between the readings had to be extended to at least 10 min to allow a reasonable number of samples to be examined in one run However the gradient of the ascending part of the CL curve was related to the concentration of serum (Fig 4b) Using this gradient the CL activity could conveniently be expressed as Δ CPM/min This activity was also linearly proportional to the number of viable bacteria up to 2 6 10^8 in the reaction mixture Doubling this number increased CL by 22 per cent Thus to assess the opsonic activity of serum the number of bacteria has to be kept constant and results from one experiment should not be compared numerically to those of others unless an internal standard (PHS) and enumeration of viable bacteria make it permissible

PMN from 4 normal subjects showed no differences in eliciting CL. The precision of individual CL registrations was examined in 13



Fig 3 Interaction between polymorphonuclear leukocytes and meningococci in the presence of homologous convalescent serum (a) and in the absence of serum (b) after 1 h of incubation

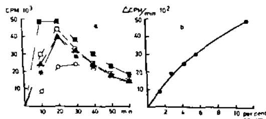


Fig 4 The relationship betweem opsonizing serum and chemiluminescence (CL) Fig 4a shows CL curves at different serum concentrations (■ 11 4 per cent (v/v) □ 5 7 per cent ▲ 4 3 per cent ● 2 8 per cent ○ 1 4 per cent) In Fig 4b the gradient of the initial ascending part of the CL curves in Fig 4a are plotted against the respective serum concentrations

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Phagocytosis and bacterial killing To visualize phagocytosis during the CL procedure 1 drop of reaction mixture was dispersed in 1 drop of albumin solution (5 g/dl) on a microscope slide spread dried fixed in methanol stained with Giemsa solution and examined microscopically The concomitant killing of bacteria was determined by transferring 0 01 ml of the reaction mixture to 1 ml distilled water to lyse PMN (6) and then diluting further in HBSS with albumin to determine the total number of surviving meningococci

RESULTS

The addition of serum to a mixture of bacteria and PMN resulted in a marked increase in CL (Fig 1) Homologous convalescent serum induced a more rapid increase and a higher peak than PHS Controls devoid of serum showed low CL counts increasing slowly with time Simultaneously a rapid and marked decrease in the number of viable bacteria occurred in the reaction mixture containing serum while controls showed only moderate changes in bacterial viability (Fig 2) In the bacterial concentration range used bactericidal activity of serum was not demonstrated Microscopic examination revealed that the addition of serum resulted in a marked ingestion of bacteria by PMN while in the

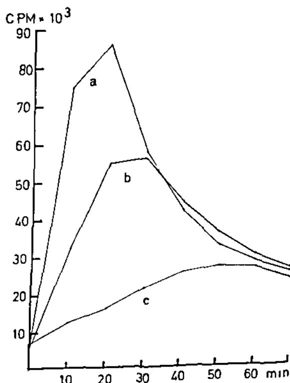


Fig 1 Chemiluminescence in reaction mixtures of polymorphonuclear leukocytes and meningococci in the presence of homologous convalescent serum (a) pooled human serum (b) and in the absence of serum (c)

assessment of opsonic activity than previous phagocytosis experiments with meningococci

The exact mechanism of CL during phagocytosis is not known but is most likely associated with oxidation of components from the ingested microorganism (2) Measurement of the initial rate of phagocytosis has been used for quantitation of opsonin induced phagocytosis of *Escherichia coli* lipopolysaccharides (8) The kinetics of the cellular events causing the initial increase in CL are unknown Nevertheless in our study this gradient correlated well to opsonic activity, and permitted the readings to be completed in less than 10–15 min

Too far reaching conclusions should not be drawn from the preliminary investigations with convalescent sera However the high CL inducing activity of a group C convalescent serum against a group B meningococcal strain and the difference between the two group B strains against serum homologous to one of them indicate that immunological properties besides group specificity are of importance in opsonization

Using a chicken embryo model *Frasch et al* (3) stated that group specific antibodies from group B meningococci showed mainly opsonizing activity while type specific antibodies acted more bactericidal than opsonizing *Zollinger et al* (9) showed that a vaccine prepared from a group C serotype 2 *Meningococcus* produced active protection against infection by a group B serotype 2 meningococcal strain in mice and induced bactericidal activity against both group C and B strains in human volunteers These findings and others leave many questions about host defence mechanisms in meningococcal disease unanswered So far the role of the various opsonizing factors has not been determined However the precision and simplicity of CL measurements seem to offer a new opportunity to study the immunological aspects of this disease

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individual replicate samples at two different levels of activity 5 10^3 and 27 10^3 CMP The standard deviations expressed as percentage of the means (coefficients of variation) were 5 8 and 6 1 respectively

Heating convalescent serum to 56 °C for 30 min reduced CL activity to 9 per cent of controls The addition of complement restored 86 per cent of the activity Complement alone showed barely detectable activity and heat inactivated complement no such activity The CL inducing activity of nonheated convalescent serum was slightly reduced by the addition of guinea pig serum (complement)

Preopsonization of bacteria with sera (active serum or heat inactivated serum plus complement) induced CL activity when opsonized bacteria and PMN were mixed but the activity was 30–50 per cent lower than when serum or inactivated serum plus complement was added to the reaction mixture Omitting complement during preopsonization reduced the CL inducing activity of heat inactivated serum to 20 per cent of controls indicating that both serum and complement were essential for opsonization

Absorbing convalescent serum with its homologous strain reduced its CL inducing activity to 35 per cent of comtrols

Preliminary observations were made with sera from patients recovering from meningitis due to other bacterial strains and from one patient with pericarditis due to group C meningococci (Table 1) The CL inducing activity of sera from convalescents of meningococcal disease was uniformly higher than that of sera from other patients and PHS One case of meningitis caused by an unindentified bacterial strain showed high CL inducing activity in serum when assayed against PMN and group B meningococci This supported the clinical impression that the patient had suffered from a meningococcal disease

Convalescent serum was examined on its CL inducing activity of PMN with either the homologous strain (12809/79 1 1 10^8 bacteria) or a heterologous strain (NCTC 10026 2 7 10^8 bacteria) In spite of the higher number of bacteria present in the latter reaction mixture the CL inducing activity was only 25 per cent of that in the reaction mixture containing meningococci and the homologous serum After repeated cultures the reactivity of the original bacterial strain (12809%79) showed a tendency to subside

TABLE 1 *Chemiluminescence Inducing Activity of Convalescent Sera (5 7 Per Cent v/v) in a Mixture (3 5 ml) of* Neisseria meningitidis *Group B (Strain 12809/79 0 7 10^9 Organisms) and Polymorphonuclear Leukocytes (0 5 10^7 Cells)*

Patient number	Convalescent serum from patient infected with	Chemiluminescence ΔCPM/min 10^3
1	Homologous strain (12809/79)	64
2	*N meningitid s* group C (16673/79)	61
3	*N meningitidis* negative cultures clinical evidence	54
4	*N meningitidis* group B (21086/79)	39
5	*S pneumoniae* (?0809/79)	25
6	*H nfl en ae* (19518/79)	23
–	P ed human serum	19

Volume adju ment by PBS mean of 2 experiments sera drawn 3 we ks after onset of disease

DISCUSSION

In the present study CL was correlated to phagocytosis *and killing of bacteria* The interlin

of PMN during phagocytosis and/or killing of t e meningococci

CL was enhanced by pretreatment of bacteria with serum and even more by the presence of serum in the reaction mixture In each procedure the CL activity depended on both thermolabile and thermostable factors The CL activity was higher in convalescent sera from patients with meningococcal disease than from control patients and was markedly but not completely absorbed by a single incubation with the homologous strain These findings are in agreement with observations in phagocytosis experiments by *Roberts* (6 7) employing group B meningococci and rabbit PMN and sera as well as group A and C meningococci and human PMN and sera However CL most probably provides a simpler and more precise method for

assessment of opsonic activity than previous phagocytosis experiments with meningococci

The exact mechanism of CL during phagocytosis is not known but is most likely associated with oxidation of components from the ingested micro organism (2) Measurement of the initial rate of phagocytosis has been used for quantitation of opsonin induced phagocytosis of *Escherichia coli* lipopolysaccharides (8) The kinetics of the cellular events causing the initial increase in CL are unknown Nevertheless in our study this gradient correlated well to opsonic activity and permitted the readings to be completed in less than 10–15 min

Too far reaching conclusions should not be drawn from the preliminary investigations with convalescent sera However the high CL inducing activity of a group C convalescent serum against a group B meningococcal strain and the difference between the two group B strains against serum homologous to one of them indicate that immunological properties besides group specificity are of [illegible]

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PREGNANCY SPECIFIC BETA-1 GLYCOPROTEIN (SP1) IN PATIENTS WITH TROPHOBLASTIC DISEASE: MOLECULAR HETEROGENEITY AND A »SP1 CONSUMING FACTOR«

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Westergaard J G Sizaret Ph Hindersson P Folkersen J & Teisner B Pregnancy specific beta 1 glycoprotein (SP1) in patients with trophoblastic disease Molecular heterogeneity and a »SP1 consuming factor« Acta path microbiol scand Sect C 88 233–236 1980

The reactivity of sera from 19 patients with trophoblastic disease was analyzed in analytic immunoelectrophoresis using rabbit anti human pregnancy specific beta 1 glycoprotein (SP1) antisera Molecular heterogeneity of SP1 was demonstrated in crossed immunoelectrophoresis and a »SP1 consuming factor« was found in serum from a patient with choriocarcinoma The »SP1 consuming factor« was precipitated by 1 65 M $(NH_4)_2SO_4$ The results are discussed in relation to quantification of circulating SP1 in patients with trophoblastic disease

Key words Trophoblastic tumours pregnancy proteins pregnancy specific beta 1 glycoprotein molecular heterogeneity

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Pregnancy specific beta 1 glycoprotein (SP1) was originally described by *Tatarinov & Masyukevich* (12) and *Bohn* (2) The clinical application of SP1 measurement has been reviewed and the data suggest that SP1 quantification will be a useful marker in different pathological conditions in pregnancy (3 4 5) Recently SP1 has been proposed as an additional marker to human chorionic gonadotropin (HCG) in the management of patients with trophoblastic disease (1 8 9 13)

SP1 has been characterized as a beta 1 glycoprotein with a molecular weight of 90 000 daltons Recently however material immunochemically identifiable with SP1 has been shown to be physicochemically heterogeneous in serum from pregnant women and an alphomobile protein with SP1 determinants and a molecular weight of 400 000 Daltons has been described (14 15, 16) In the present study circulating SP1 in sera from patients with trophoblastic disease were examind by analytic immunoelectrophoresis in order to establish whether the molecular heterogeneity earlier demonstrated in pregnancy occurred also in the former patient sera

MATERIALS AND METHODS

Sera

19 serum samples drawn from 19 patients with trophoblastic disease were analysed All samples were stored at −20 °C 13 samples were drawn from women with hydatidiform mole (12 pretreatment and one posttre[illegible]
(one [illegible]
was [illegible]

Crossed Immunoelectrophoresis

Crossed immunoelectrophoresis was performed in 1% Indubiose A-37 (L'Industrie Biologique Francaise, Clichy, France) using thris-barbital buffer, ionic strength 0 02, pH = 8 6 The first dimensional electrophoresis was run at 10V/cm until a bromphenol blue stained albumin marker had migrated 2 cm The antibody-containing second dimensional gel contained 1 µl anti SP1 (Code A130, lot 018c, DAKO Immunoglobulins, Copenhagen, Denmark) per cm^2 and 4% Polyethylene Glycol 6000 (PEG) to enhance the precipitate in the alpha region (15, 16)

Line Immunoelectrophoresis

Line immunoelectrophoresis was performed as described by *Krøll* (6) with the same gel buffer system as described for crossed immunoelectrophoresis Four gel strips were moulded on a 10 × 10 cm glass plate, from the cathodic side (Fig 2), A a neutral gel (10 × 1 × 0 15 cm), B an antigen containing gel (10 × 0 5 × 0 15 cm) which received 14 µl late pregnancy serum per ml agarose, C a neutral gel (10 × 1 × 0 15 cm) with 9 wells cut and D the antibody containing gel (10 × 7 5 × 0 15 cm) with 0 5 µl anti SP1 (DAKO immunoglobulins, Copenhagen Denmark) per cm^2 Thewells in C received 15 µl samples each and the electrophoresis was run at 2 5 V/cm for 18 h

$(NH_4)_2SO_4$ Preparation

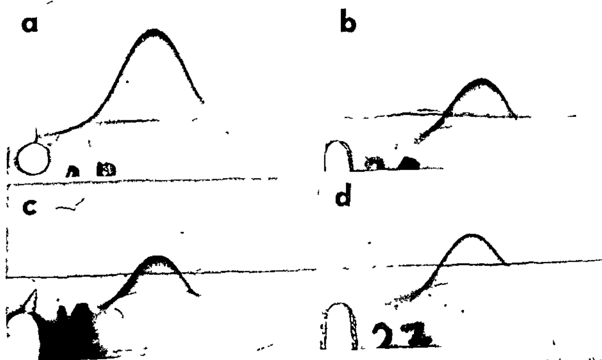
250 mg $(NH_4)_2SO_4$ was added to 1 ml serum After 4 h at room temperature the supernatant and precipitate were separated by centrifugation at 4000 *g* for 30 minutes The precipitate was washed three times with 1 65 M $(NH_4)_2SO_4$ and dissolved in H_2O The dissolved precipitate and the supernatant were dialysed over night against electrophoresis buffer and tested in line immuno electrophoresis

RESULTS

Serum samples from the nineteen patients with trophoblastic disease were analyzed in line immunoelectrophoresis Using this technique SP1 antigen was detectable in all patients with hydatidiform mole In the serum samples drawn from patients with choriocarcinoma SP1 antigen was only detectable in one out of six patients This sample was drawn before the treatment was initiated

The SP1 positive samples were analyzed further in crossed immunoelectrophoresis and a beta mobile and an alpha mobile SP1 fraction were demonstrated (Fig 1) In only one sample, a pretreatment sample from a patient with hydatidiform mole, the alpha mobile SP1 was not observed (Fig 1 C)

Fig 2 shows the analysis of 8 serum samples in line immunoelectrophoresis Wells no 2, 3 and 6 received samples which caused positive deflection of the line precipitate indicating the presence of SP1 antigen in the samples However, one of the samples tested (Fig 2, well no 5) caused a significantly negative deflection of the line precipitate indicating



... form mole (a c d) and
gel In 3 samples

(a b d) a diphasic precipitation pattern



Fig 2 Line immunoelectrophoresis of serum samples from patients with hydatidiform mole (wells no 2 4 6) and choriocarcinoma (wells no 1 3 5 7 8) Well no 9 received buffer only A negative deflection of the line precipitate indicating a »SP1 consuming factor« in the sample is seen in well nr 5

the presence of an SP1 antigen binder in the serum this serum was a posttreatment sample obtained from one of the patients with choriocarcinoma The »SP1 antigen binder« was completely precipitated by 1 65 M $(NH_4)_2SO_4$ and recovered from the washed redissolved and dialyzed precipitate

DISCUSSION

Recently pregnancy specific beta 1 glycoprotein (SP1) has been proposed as an additional marker for residual tumour growth in patients with trophoblastic disease (1 8 9) However the recent observations on the molecular heterogeneity of SP1 in pregnancy serum casts some doubt on any observations in which SP1 is quantified by immunochemical techniques if this molecular heterogeneity is not taken into account (10 11 15 16)

The results presented here show that molecular heterogeneity of circulating SP1 is not unique to pregnancy but also seen in sera from patients with trophoblastic disease Like in pregnancy (16) the ratio between the beta and alpha mobile SP1 fraction differs markedly from patient to patient and in one patient (Fig 1 C) the alpha mobile fraction could not be visualized by conventional crossed immunoelectrophoresis

Furthermore serum drawn post treatment from one patient with choriocarcinoma contained a »SP1 consuming factor« This »factor« the nature of which is still unknown has an electrophoretic mobility slower than SP1 (beta 1) as indicated by the line immunoelectrophoresis data In addition the »factor« was completely precipitated by 1 65 M $(NH_4)_2SO_4$ and these observations are compatible with the suggestion that the »factor« is an autoantibody produced against an altered SP1 antigen produced by the malignant cell However the possibility that the »SP1 binder« is a SP1 receptor molecule or a SP1 reactive enzyme present in the circulation has to be taken into consideration

Using double antibody radioimmunoassay *Rutanen & Seppälä* (7) recently reported that 17% of the serum samples drawn from choriocarcinoma patients after one year in remission and with good prognosis were found SP1 positive whereas they were HCG negative However, any »SP1 consuming factor« in a serum sample forming complex with the radiolabelled SP1 may mask antigenic determinants and if the complex is not precipitated by the second antibody this could give a false positive reaction

Further investigations are required before the significance and the precise nature of the »SP1 binder« can be elucidated

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IMMUNOGLOBULIN CLASSES OF URINARY AND SERUM ANTIBODIES IN MYCOPLASMAL PYELONEPHRITIS

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Ernø H & Thomsen A C Immunoglobulin classes of urinary and serum antibodies in mycoplasmal pyelonephritis Acta path microbiol scand Sect C 88 237-240 1980

During 9 attacks of *M hominis* pyelonephritis in 8 patients the presence of IgA IgG and IgM antibodies in urine and serum was studied by an indirect immunofluorescence technique Antibodies were present in the urine for a period of 12 days One to 4 days after the onset of the attack IgA was in all cases demonstrable in urine followed later by IgG IgM was present in the urine of one patient only during the first 4 days of illness In serum IgG appeared as early as 1-4 days after the attack followed later by IgA and IgM IgA disappearing after one to two weeks

Key words Immunoglobulin classes mycoplasma pyelonephritis

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Mycoplasmas of the species *Ureaplasma urealyticum* and *Mycoplasma hominis* are common inhabitants of the urethra and have also been isolated from bladder urine aspirated by suprapubic puncture (1 4 14) and from the upper urinary tract (5 14)

U urealyticum has been associated with urethritis but the question of a causal relationship is still in dispute However there is growing evidence for the point of view that ureaplasmas are causing a proportion of nongonococcal urethritis (8) whereas no association has been demonstrated between cystitis and any mycoplasmal infection

M hominis was isolated from the upper urinary tract of patients with symptoms of acute pyelonephritis but not from patients with non infectious urinary tract diseases (9 10) Antibodies to *M hominis* measured by indirect hemagglutination (IHA) were demonstrable in serum and in ureteric and bladder urine from some of these patients (11) The appearance of antibodies to *M hominis* in urine was found to be of a high diagnostic value (8) and the data suggest that *M hominis* causes some cases of pyelonephritis whereas there is no indication for a similar role of ureaplasmas

The antibody response to other mycoplasmal infections in humans and animals is very often used for diagnostic purposes It is difficult however to interpret serum antibody responses to those mycoplasmas which are commensals and considered only potentially pathogenic unless very clear changes in titre are observed A serological diagnosis may therefore sometimes be more reliable when local antibodies are demonstrated As examples arthritis in pigs (13) and mastitis in cows (2) are mentioned

The purpose of the present study was to confirm by use of the indirect immunofluorescence technique (IMF) the presence of antibodies to *M hominis* in urine which were demonstrated earlier by the IHA test (12) Furthermore the distribution of specific antibodies within IgA IgG and IgM immunoglobulin classes was studied

MATERIAL AND METHODS

Eight patients previously described with 9 acute attacks of pyelonephritis caused by *M hominis* alone or in combination with bacteria were selected (12) The diagnosis of an acute attack of pyelonephritis was based

on the presence of acute lumbar pain, fever, and pyuria The onset of the attacks was defined as the day of the first appearance of one of these symptoms In five of the patients it was the first attack of urinary tract infection, and the level of serum creatinine was normal The remaining three patients suffered from chronic pyelonephritis with obstruction, as estimated by a history of previous urinary infections, increased serum creatinine, and intravenous pyelography The involvement of *M hominis* was established by cultivation from ureteric or bladder urine of *M hominis* alone in 7 attacks and together with bacteria in two attacks (Nos 251 and 414, second attack) concurrent with the occurrence of a significant rise in serum antibodies in some cases and the appearance of urine antibodies in all cases (12)

Serum samples and midstream urine specimens were obtained for antibody examination Immediately after collection the specimens were examined for antibodies to *M hominis* by IHA The test was performed with glutaraldehyde fixed sheep erythrocytes A pooled antigen, prepared from 3 strains of *M hominis* was used as antigen (12) After storage at −20 °C for 2–6 months the specimens were reexamined for antibodies by IHA as well as IMF (6) For the IMF test urine was tested undiluted whereas serum was diluted 1 10 and 1 20 in phosphate buffered saline pH 7 2 (PBS) Unfixed colonies of strain P7 of *M hominis* (10) were incubated with a drop of the test sample for 30 min at 22 °C in a moist chamber The blocks were washed 2 × 10 min with PBS, incubated (30 min) with a drop of fluorescein-isothiocyanate conjugated antiserum against either human IgA IgM or IgG (Wellcome) and washed 2 × 10 min in PBS Serum containing IgA IgG and IgM antibodies to *M hominis* was used through out the study as a positive control Likewise serum and urine without specific antibodies, as measured by IHA, served as negative controls The fluorescence was read by means of a Zeiss standard (RA) microscope with incident illumination

RESULTS

By IHA performed immediately after collection and by IMF after storage at −20 °C for 2 to 6 months antibodies were demonstrated in serum during 7 of the attacks and in urine samples representing all 9 attacks (Table 1) After storage, however antibodies in urine could be demonstrated by IHA in one case only (No 611), while serum antibodies were still demonstrable

Immunoglobulin classes in urine In all 9 cases fluorescence for IgA was observed during the first 4 days of the attack, in some patients up to as late as 12 days after the onset of the disease Antibodies of the IgG class were also demonstrated in all 9 attacks, concurrent with or after the appearance of IgA antibodies and likewise not later than 12 days after onset of the disease Antibodies of the IgM class were only present in one patient and only during the first 4 days of illness In this case, both *M hominis* and bacteria were incriminated in the disease

Immunoglobulin classes in serum In 2 attacks (Nos 611 and 634), characterized by mild symptoms, it was not possible to demonstrate antibodies by any of the tests employed Of the remaining 7 cases IgA was demonstrated in 6 cases, between the 5–8 day of the disease and never later than 12 days after onset IgG was observed in all 7 cases In 3 instances IgG was present already 1–4 days after the onset of the disease IgG persisted in the serum of all 6 patients examined throughout the period of observation (21 to 84 days) IgM was present in 6 cases, appearing from day 5 to day 12 and persisted with one exception for the same period of time as IgG

DISCUSSION

The results of the present IMF study of antibodies to *M hominis* in urine and serum from patients with acute attacks of pyelonephritis confirm the presence of urine and serum antibodies to *M hominis*, as previously demonstrated by IHA (12) However, urine antibodies were rarely found by IHA after storage for 2–6 months at −20 °C, while they still could be detected by immunofluorescence The presence of urea and leucocytic enzymes in urine may lead to a partial degradation of the antibody molecule, which might impair the agglutinating ability, but leave the antigen combining capacity of the molecule intact (3) This might explain the immunofluorescence test being positive and the IHA being negative after storage of the urine samples for several months

In urine, antibodies of immunoglobulin class A were always found to be part of the first demonstrable immune response to *M hominis* infection of the upper urinary tract, either alone or in combination with IgG and IgM In serum, IgG was always part of the early immune response and persisted throughout the period of observation

An inflammatory process of the kidney makes possible a leakage of serum antibodies into the urine However the urine antibodies seem, at least partly, to be synthesized locally, because they often appeared earlier than in serum and in 2 cases antibodies were not present at all in serum A final proof of local IgA antibody synthesis might have been achieved by using fluorescein-conjugated anti-secretorycomponent antibodies

The finding that locally occurring antibodies may not parallel the serum antibody response was also demonstrated in experimental pyelonephritis in rats (7) The sequence may depend on the antigens and the severity of the lesions, as it was found in a study

TABLE 1 *Occurrence of Antibody in Urine and Serum of 8 Patients with 9 Acute Attacks of Pyelonephritis*

Patient No		Immunofluorescent immunoglobulin differentiation and titres of antibody as measured by IHA							
		Urine				Serum			
		Days after onset of disease							
		1–4	5–8	9–12	>12	1–4	5–8	9–12	13–60
10	IgA	+	NT[a]	–	–	NT	NT	+	–
	IgM	–	NT	–	–	NT	NT	+	+
	IgG	–	NT	+	–	NT	NT	+	+
	IHA	1024	NT	16	<16	NT	NT	128	256
136	IgA	+	+	+	–	–	+	–	–
	IgM	–	–	–	–	–	+	+	+
	IgG	+	–	+	–	–	+	+	+
	IHA	64	128	32	<16	<16	32	512	512
154	IgA	+	+	+	–	NT	+	–	–
	IgM	–	–	–	–	NT	+	+	+
	IgG	–	+	+	–	NT	+	+	+
	IHA	32	256	256	<16	NT	32	64	512
251	IgA	+	–	+	NT	–	+	+	NT
	IgM	+	–	–	NT	–	+	+	NT
	IgG	+	–	+	NT	+	+	+	NT
	IHA	64	<16	64	NT	16	32	256	NT
414[b] first attack	IgA	+	NT	+	–	–	NT	+	–
	IgM	–	NT	–	–	–	NT	+	+
	IgG	–	NT	+	–	+	NT	+	+
	IHA	128	NT	32	<16	32	NT	256	256
414[b] second attack (2 months later)	IgA	+	NT	+	–	–	NT	+	–
	IgM	–	NT	–	–	–	NT	+	+
	IgG	+	NT	+	–	+	NT	+	+
	IHA	64	NT	64	<16	512	NT	256	512
611[c]	IgA	+	+	–	–	–	–	–	–
	IgM	–	–	–	–	–	–	–	–
	IgG	–	+	–	–	–	–	–	–
	IHA	16	512	<16	<16	<16	<16	<16	<16
634	IgA	+	+	–	–	NT	–	–	–
	IgM	–	–	–	–	NT	–	–	–
	IgG	–	+	–	–	NT	–	–	–
	IHA	32	32	<16	<16	NT	<16	<16	<16
701	IgA	+	+	+	–	NT	–	–	–
	IgM	–	–	–	–	NT	–	–	–
	IgG	+	+	+	–	NT	–	+	+
	IHA	64	128	64	<16	NT	<16	32	128

[a] NT Not tested

[b] This patient had suffered from several previous attacks of urinary tract infections and had two attacks during the period of observation

[c] [illegible] t of the disease

of pyelonephritis caused by *E coli* (15) that IgA antibodies were observed in the urine during the first days of infection but not earlier than IgG antibodies and in some cases even after IgG Urine IgM was in these examinations also only occassionally demonstrable Urine antibodies and serum IgA decreased somewhat later than observed in our study, possibly because of a longer period needed for the healing of pyelonephritic lesions caused by *E coli* as compared to a mycoplasmal inflammation In mycoplasmal bovine mastitis due to inoculation of *M bovigenitalium* IgA was likewise present in serum during the acute attack only (2) and the appearance of IgA in the milk was the first demonstrable antibody response

Differences between the local and serum antibody response may also reflect the fact that the immune response in serum seen in relation to cases of local inflammatory processes depend very much on earlier stimulation by the causative agent Earlier stimulation is a possibility in cases of diseases caused by *M hominis* as this microorganism in general appears to be a commensal frequently occurring in humans Whether the antibodies produced locally are of more importance than serum antibodies in terms of curative effect is not clear, but it is worthwhile noting that only urine antibodies were present in 2 patients with mild pyelonephritis of short duration and that the high level of serum antibody in patient No 414 did not prevent a second attack It is generally believed that antibodies in mycoplasmal diseases are the prime mediators of resistance and especially local antibodies correlate with protective immunity As very little is known of the mechanism by which *M hominis* cause disease either alone or together with other pathogens or debilitating factors it is difficult to hypothesize on the function of the urinary antibodies Clearly in ascending infections local antibodies may prevent adsorption of the microorganisms to the surface epithelium neutralize toxins and enzymes prevent penetration of soluble antigens or exert mycoplasmacid or mycoplasmastatic effect The local antibodies may be of less importance in haematogeneous infection a possibility which exists in cases of mycoplasmaemia especially combined with obstruction or surgery of the genitourinary tract

It is concluded that specific antibodies are demonstrable in the urine of patients with mycoplasmal nephritis using the indirect immunofluorescence technique This method may therefore contribute to an etiologic diagnosis of inflammatory processes of the kidneys

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Acta path microbiol scand Sect C 88 241-245 1980

A SIMPLE METHOD FOR THE PRODUCTION OF F(ab')$_2$ PREPARATIONS BY PEPSIN DIGESTION OF TOTAL SERUM PROTEIN

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Poulsen F & Hjort, T A simple method for the production of F(ab')$_2$ preparations by pepsin digestion of total serum protein Acta path microbiol scand Sect C 88 241-245 1980

An easy and rapid method for producing F(ab')$_2$ fragments by pepsin treatment of total serum protein is described No further purification of the hydrolysate was used Most serum proteins were degraded to small peptides which were removed by dialysis the preparations thus being relatively rich in F(ab')$_2$ fragments The degree of hydrolysis was determined by means of an antiserum against the part of the γ-chain which is degraded by pepsin Under optimal conditions for hydrolysis unsplit IgG could not be detected by double immunodiffusion Using sperm agglutinating and immobilizing sera the F(ab')$_2$ preparations could be characterized functionally After hydrolysis the agglutinating activity was relatively unchanged whereas the immobilizing activity had vanished

Key words F(ab')$_2$ fragments total serum protein sperm antibodies

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The use of F(ab')$_2$ fragments may in certain immunological test systems be advantageous to the use of intact IgG antibodies mainly because non specific binding through the Fc part of the IgG molecule can be avoided Most commonly F(ab')$_2$ preparations are produced by pepsin digestion of purified IgG and isolation of the F(ab')$_2$ fragments by gel filtration as done by e g *Jaquet & Cebra* (1965) Such procedures as well as the simplified method of *Madsen & Rodkey* (1976) using digestion of whole globulin fraction are quite laborious and time-consuming and therefore not suited for preparing F(ab')$_2$ fragments from large series of sera

Faced with the task of producing F(ab')$_2$ preparations from a considerable number of sera with sperm agglutinating and immobilizing antibodies we have therefore investigated the possibility of preparing F(ab')$_2$ fragments of satisfactory quality just by pepsin digestion of total serum protein With

Immunochemical charaterization was performed by determining the reduction or disappearance of the C_H2 part of the heavy chain which is degraded by pepsin during its cleavage of the IgG molecule into F(ab')$_2$ and pFc Immunobiological characterization could be obtained by comparing the sperm agglutinating and immobilizing activity of the preparations Previous experiments with purified F(ab')$_2$ fragments have revealed that the agglutinating activity is essentially unaffected by the pepsin digestion whereas the immob

... et al 1976)

MATERIALS AND METHODS

Sera

Human sera with high titres of sperm agglutinating and immobilizing antibodies were obtained from vasectomized men and from an infertile man (119). By absorption of the sera with protein A producing *Staphylococcus aureus* the agglutinating activity in three sera (sera Nos 116, 77 and 95) could be totally removed and in two sera (Nos 110 and 119) the agglutination titre was reduced to 8 from 256 and 1024, respectively, the antibodies thus belonging mainly to the IgG class.

Antisera

Antiserum against the part of the γ chain situated between the F(ab')$_2$ part and the pFc' part of the molecule was produced in rabbits by immunization with Fc fragments. The Fc fragments were prepared as described by *Sanderson & Lanning* (1970) and dissolved to a concentration of 0.4 mg/ml. The immunization was done by giving three rabbits an intramuscular injection of a mixture of 250 µl Fc solution and 250 µl complete Freund's adjuvant on days 0, 6 and 70. Serum was taken 26 days after the last injection. The most potent of the antisera was rendered specific for the region of interest by successive passages over immunosorbent columns of Sepharose 4B containing covalently bound F(ab')$_2$ fragments and pFc' fragments, respectively. The specificity was confirmed by Ouchterlony immunodiffusion against IgG, F(ab')$_2$ and pFc'. Since the C_H2 domain belongs to the particular part of the γ-chain, this antiserum is in the following referred to as anti »C_H2«.

Rabbit antiserum against human IgG Fd fragment was from Behringwerke AG, Germany (anti Fd). Rabbit anti human IgG specific for Fc fragment (anti Fc) and rabbit anti human serum protein (anti NHS) were obtained from Dakopatt A/S, Denmark.

Tests for Sperm Antibody Activity

Sperm agglutinins were demonstrated by the gelatin agglutination technique (*Rose et al.* 1976) and by the tray agglutination technique as used by *Ingerslev* (1979). Immobilizing activity was determined according to the method of *Isojima et al.* (1968) with the modification that titration was done. The titre was defined as the greatest sample dilution in which at least half of the initially motile spermatozoa were immobilized in the presence of complement.

With the different techniques all samples from a given experiment were tested in the same batch.

Determination of Unsplit IgG

Unsplit IgG was detected by double immunodiffusion with the anti »C_H2« antiserum. The IgG region between the F(ab')$_2$ part and the pFc' part is degraded during pepsin hydrolysis of IgG and precipitates produced with the anti »C_H2« will consequently indicate the presence of unsplit IgG (whereas anti Fc could react also with pFc'). The amount of unsplit IgG was determined by testing a series of sample dilutions. The reciprocal of the highest dilution giving a precipitation line was used as a semiquantitative measure of the amount (precipitation titre).

Gel Filtration

was carried out on a column of Sephadex G 200 (Pharmacia) (2.6 × 85 cm) equilibrated with 0.1 M Tris HCl pH 8.0 containing 0.2 M NaCl. The flow rate was 20 ml/h.

Immunodiffusion and Immunoelectrophoresis

Double immunodiffusion was performed in 1 per cent agarose gel (Litex, Denmark) using 0.15 M NaCl buffered with 0.01 M phosphate pH 7.2 (PBS). Immunoelectrophoresis was carried out according to a modification of the method of *Scheidegger* (1955) in 1 per cent agarose in Tris veronal buffer pH 8.6.

Protein Determination

Protein concentrations were determined by the biuret method as described by *Doumas* (1975). Human albumin (A grade, Calbiochem, California) was used as standard.

Pepsin Digestion

Equal volumes of 0.2 M acetate buffer of the desired pH and serum were mixed and pH adjusted by addition of a few drops of 1 M HCl. This serum protein mixture was then incubated with pepsin (three times crystallized [illegible]

amount of precipitate formed during incubation and the reaction was stopped by addition of solid Tris to pH 8. Finally, the digest was dialyzed against PBS.

RESULTS

Optimal Conditions for Pepsin Hydrolysis

Table 1 shows the results of one experiment with variation of the digestion conditions, i.e. the E/S ratio, the pH and the time. Only pH 4.5 and 4.0 were tested because these values are within the pH range commonly used for production of F(ab')$_2$ fragments. The E/S ratios were based on the protein concentration determined by the biuret method.

The different digestion conditions gave preparations with an unchanged titre in the gelatin agglutination test. The tray agglutination titre was

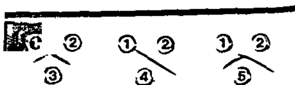


Fig. 1. Ouchterlony immunodiffusion of hydrolysate (1) and a corresponding dilution of serum (2) against anti Fd (3), anti »C_H2« (4) and anti Fc (5).

TABLE I *Agglutination Immobilization and Precipitation Results of Hydrolysates of Serum B 116 Prepared by Pepsin Digestion under Different Conditions*

Digestion conditions			Gelatin agglutination	Tray agglutination	Immobilization	Precipitation
pH	Time (h)	E/S ratio	Titre	Titre	Titre	[b]Titre
Untreated serum			256	128	32	512
4 0	10	1/100	256	128	<2[a]	16
4 0	10	2/100	256	128	<2	4
4 0	10	4/100	256	128	<2	2
4 0	20	1/100	256	128	<2	<2
4 0	20	2/100	256	256	<2	<2
4 0	20	4/100	256	128	<2	<2
4 5	10	1/100	256	128	8	256
4 5	10	2/100	256	256	4	128
4 5	10	4/100	256	64	<2	128
4 5	20	1/100	256	256	<2	128
4 5	20	2/100	256	256	<2	32
4 5	20	4/100	256	128	<2	16

[a] <2 indicates negative reaction in lowest dilution tested (1 2)
[b] Reciprocal of highest dilution giving precipitation with anti »C_H2«

relatively constant with some small variations apparently without correlation to the digestion conditions In contrast both the immobilization titres and the precipitation titres with anti »C_H2« were changed drastically during the digestion with three combinations (pH 4 0 20 h and E/S = 1/100 2/100 and 4/100) in which neither immobilizing nor precipitating activity could be detected These conditions are optimal for making F(ab)$_2$ preparations which retain the sperm agglutinating activity but are free of the Fc fragment activities An E/S ratio of 2/100 a pH of 4 0 and a digestion time of 20 h at 37 °C was chosen and used in all further digestions

Characterization of the Hydrolysate

The hydrolysate prepared under optimal conditions (specified above) was analysed by Ouchterlony immunodiffusion gel filtration and immunoelectrophoresis

Fig 1 illustrates that the hydrolysate as well as the serum gave precipitation lines in double immunodiffusion with anti Fd and anti Fc However with anti »C_H2« only untreated serum gave a precipitation line the lack of reaction of the hydrolysate indicating that the »C_H2« part of the IgG molecule was degraded during pepsin treatment [illegible] formation of a spur

Fig 2 shows the gel filtration pattern of the

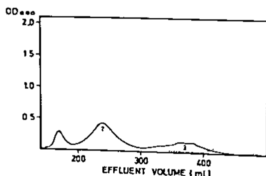


Fig 2 Gel filtration on Sephadex G 200 of 5 ml hydrolysate with 5 ml of a corresponding dilution of serum (1 2) as reference
——— hydrolysate = serum

hydrolysate This indicates that pepsin degraded a considerable part of the serum protein to dialysable material and that the remaining protein could be resolved into 3 peaks Peak 2 with an elution position just after the IgG peak contained material which gave reaction with anti Fd in double immunodiffusion (F(ab)$_2$) and peak 3 contained material which gave reaction with anti Fc (pFc)

From the immunoelectrophoresis of the hydrolysate with anti NHS shown in Fig 3a it appears that the number of precipitin arcs was greatly reduced as compared with that of serum The precipitin arc of lowest mobility on the figure represents both anti Fd and anti Fc reactive material

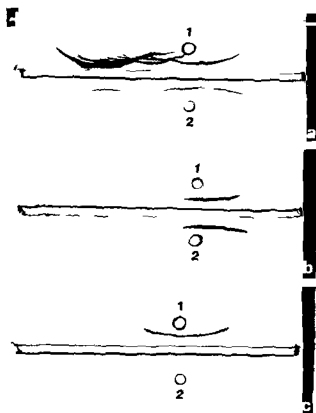


Fig 3 Immunoelectrophoresis of a 1 2 dilution of serum (1) and hydrolysate (2) Developing antiserum was anti NHS (a) anti Fd (b) and anti Fc (c)

as shown by using anti Fd and anti Fc respectively as developing antiserum (Fig 3b and c)

Application of the Method to Different Sera

The wide range of E/S ratios i e 1/100–4/100 giving F(ab')$_2$ preparations without detectable unsplit IgG means that the same concentration of enzyme can be used with a wide range of different substrate concentration The total protein concentration in serum varies relatively less (65–80 mg/ml) Therefore if a mean value of 73 mg/ml is used in calculating the pepsin concentration giving an E/S ratio of 2/100 the real E/S ratio will always be within the optimal range Thus the same enzyme concentration can be used for all sera and no determination of protein concentration in serum is necessary Table 2 shows the results of digestion of 5 different sera under these routine conditions i e pH 4 0 20 h of incubation at 37 °C and an E/S ratio of 2/100 based on a protein concentration of 73 mg/ml The gelatin agglutination titre was for all hydrolysates reduced by one titre step as compared with the corresponding serum whereas the tray agglutination titre showed no systematic differences between the hydrolysate and the serum Both the immobilization and the precipitation titre were in all cases reduced to undetectable levels

DISCUSSION

The present study has established the optimal conditions for the pepsin cleavage of IgG by digesting total serum protein It was found possible to produce F(ab')$_2$ fragments which had retained the antigen binding activity (as determined by the sperm agglutinating activity) and which were at the same time free of unsplit IgG to such an extent that it could not be detected by precipitation Because the agglutination titre remained unchanged or only slightly changed during all the different digestion conditions tested the optimal conditions could be chosen solely from the degree of splitting of IgG A pH of 4 0 20 h of incubation and an E/S ratio between 1/100 and 4/100 were found optimal E/S 2/100 was chosen in order to allow some variation in protein concentration without making adjustment of the amount of pepsin required

The complete fusion of the lines of hydrolysate

TABLE 2 *Agglutination Immobilization and Precipitation Results of Hydorlysates of 5 Different Sera and Corresponding Untreated Sera*

Serum No	Gelatin agglutination Titre		Tray agglutination Titre		Immobilization Titre		Precipitation [b]Titre	
	Serum	F(ab')$_2$	Serum	F(ab')$_2$	Serum	F(ab')$_2$	Serum	F(ab')$_2$
B 77	256	128	128	256	16	<2[a]	512	<2
B 95	256	128	128	64	16	<2	256	<2
B 110	256	128	256	512	128	<2	512	<2
B 116	512	256	64	256	32	<2	512	<2
B 119	2048	1024	1024	256	64	<2	512	<2

[a] <2 indicates negative reaction in lowest dilution tested (1 2)
[b] Reciprocal of highest dilution giving precipitation with anti $\gamma C_{H}2$

and serum against anti Fd the spur formation of the line of hydrolysate and serum with anti Fc and the lack of a precipitation line with hydrolysate against anti »CH2« indicate that during pepsin digestion of total serum protein the IgG molecules were split in the same way as when pure IgG is digested by pepsin resulting in production of $F(ab')_2$ fragments and pFc' fragments and degradation of the region between $F(ab')_2$ and pFc' to low molecular weight components (*Heimer et al* 1967 *Bennich & Turner* 1969) This was confirmed by the positions in gel filtration and the electrophoretic mobilities of the anti Fd and anti Fc reactive material in the hydrolysate corresponding to those of $F(ab')_2$ fragments and pFc' fragments (*Turner & Bennich* 1968) The serum protein picture as evaluated from gel filtration and immunoelectrophoresis changed with a reduction in both the number of different proteins and the total amount of protein with a considerable part of protein degraded to dialysable material

The 5 $F(ab')_2$ preparations produced with an E/S ratio of 2/100 based on a protein concentration of 73 mg/ml all showed a one titre step decrease in gelatin agglutination as compared with untreated serum This is within the variability of the technique but because of the fact that the $F(ab')_2$ preparation in all cases gave a lower titre than the serum the decrease is probably significant and due to either degradation of $F(ab')_2$ fragments or general loss of material during manipulations Another possibility might be that $F(ab')_2$ fragments are not as efficient in agglutinating cells as unsplit IgG either because the Fc fragment may play a similar role in agglutination as it does in precipitation (*Møller* 1979) or because the presence of Fc binding proteins in serum may increase the agglutinating activity of unsplit IgG Experiments done with E/S = 0 i e without pepsin also showed a decrease in titre as compared with untreated serum indicating that the decrease in titre may be due to general loss of material Taken together with the results from Table I where there was no significant decrease in titre in any of the hydrolysates and the fact that none of the 5 $F(ab')_2$ preparations could immobilize the spermatozoa or react with anti »CH2« it may be concluded that the method is suitable for preparing $F(ab')_2$ preparations which retain immunological activity and are essentially free of unsplit IgG

Compared with other methods for $F(ab')_2$ preparation the present procedure has advantages Simple and common equipment is used only small amounts of serum are needed and $F(ab')_2$ fragments can be made from many sera with a minimal investment of time

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NON-ANTIBODY COMPONENTS IN HUMAN MILK INHIBIT *ESCHERICHIA COLI* HEAT LABILE ENTEROTOXIN MEASURED BY AN ENZYME-LINKED IMMUNOSORBENT ASSAY

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Otnæss A B & Halvorsen S Non antibody components in human milk inhibit *Escherichia coli* heat labile enterotoxin measured by an enzyme linked immunosorbent assay Acta path microbiol scand Sect. C 88 247 253 1980

Milk from 11 Norwegian women was fract on ad h
chromatography
coated on microt
detected when the
immunoglobulin nature with an apparent molecular weight of >400000 in gel filtration experiments

Key words Human milk *E coli* heat labile enterotoxin ELISA

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Human milk has been reported to protect children from gastrointestinal infections (7 13) and to contain several antimicrobial factors which include immunoglobulins as well as other substances (8 15)

Enterotoxigenic strains of *E coli* may produce a heat labile toxin (LT) and/or a heat stable toxin (ST) both of which may cause diarrhoea in children and adults (17) Toxin producing *E coli* has been associated with outbreaks of gastroenteritis in neonatal nurseries (1 6 9 21)

Stol ar et al (20) reported that Guatemalan and North American human milk was able to neutralize *V cholerae* enterotoxin and/or *E coli* toxins the neutralizing activity was associated with the secretory IgA of the milk Recently *Simhon et al* (19) reported the presence of specific IgA against cholera toxin in milk from Costa Rican women *Holmgren et al* (10) reported that while milk from Pakistani women contained secretory IgA against *E coli* heat labile enterotoxin such antibodies were not detected in most of the Swedish milk samples

We wanted to study the effect on *E coli* enterotoxins of specific and non specific factors in human milk from Norway where enterotoxigenic diarrhoea in children has been suspected but only recently proven (unpublished data *Otnaess Eng & Halvorsen)*

MATERIALS AND METHODS

su pita e supernatants of 3 milk samples

were gel filtered The fractions within each protein peak were pooled and concentrated before testing

Preparation of E coli *Toxins*

E coli strain 408 3 078 H12 was kindly provided by Dr *R B Sack* Baltimore Md USA The bacteria were grown for 24 h at 37 °C in trypticase soy broth at 150 rev/min (17) and both heat labile toxin (LT) and heat stable toxin (ST) were produced The supernatant was collected after centrifugation (40000 × g for 15 min at 4 °C) and used directly for measurement of ST Crude LT was prepared by dialysis of the bacterial supernatant against saline (4 × 4 l) and lyophilized Stock solution of 1 8 mg/ml (in saline) was kept at −20 °C and diluted further in saline containing 0 5 mg/ml bovine serum albumin immediately before use In some experiments crude LT kindly provided by Dr *R B Sack* was used and identical results were obtained

Test of Toxin Activity

Heat stable toxin (ST) was measured by the infant mouse assay (4) Heat labile toxin (LT) was measured by a miniculture modification (18) of the Y1 adrenal tumour cell culture assay described by *Donta & Smith* (5) or by ELISA (21) Briefly the ELISA test was performed on polyvinyl microtiterplates coated with high titered anti serum to *V cholerae* toxin (1 2000) prepared in burro (2) (kindly provided by Dr *J B Robbins* Bureau of Biologics Bethesda Md USA) and the plates were incubated with 1) the toxin sample (incubated with milk or buffer) 2) anti LT toxin prepared in rabbit (kindly provided by Dr *B P Berdal* Norwegian Defence Microbiological Laboratory Oslo or prepared in our laboratory) 3) anti rabbit IgG prepared in goat and coupled to alkaline phosphatase (prepared by Dr *B P Berdal*) or anti rabbit IgG prepared in swine and coupled to alkaline phosphatase (Orion Helsinki Finland) and 4) the substrate sodium para nitrophenyl phosphate (Sigma Chemical Co St Louis Mo USA) The reaction was stopped after 10–30 min by the addition of 0 05 ml 2N NaOH Aliquots (100 µl) were diluted with 700 µl 0 1 N NaOH and the colour read at 400 nm The crude LT stock solution was diluted to 0 18 g/l to give OD_{400} − 0 3–0 4 in the ELISA test (100 % toxin) Upon further dilution a linear standard curve was obtained

Incubation of Toxin with Milk Fractions

Milk fractions were diluted in 0 01 M phosphate buffer pH 7 4 and aliquots (75 µl) were incubated with toxin (75 µl) for 18–24 h at 4 °C or for 2 h at room temperature Duplicate aliquots of 50 µl were tested for toxin activity in the ELISA test For the adrenal cell test the incubation mixture was diluted 3 fold immediately before the addition of duplicate aliquots (50 µl) to the adrenal cell cultures grown in microtiterplates Controls with either only toxin or milk fractions were always included

Secretory IgA and *lactoferrin* were measured by rocket immunoelectrophoresis as described previously (16)

Polyacrylamide gel electrophoresis (PAGE) was performed according to *Davis* (3) and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) as described by *Laemmli* (12) using high molecular weight standards from Bio Rad Laboratories (La Jolla California USA) for molecular weight determinations Cylindrical or flat gels were used The effect of protein eluted from polyacrylamide gels on LT was investigated by cutting the separating gels in 1–2 mm slices and the concentrating gels in 4–6 mm slices followed by freezing and thawing in 0 1 ml distilled water and gentle squeezing before 75 µl was incubated with an equal volume of LT and tested as described above

Affinity gels of anti IgA and anti IgM were prepared as described (16)

Protein was measured by the *Lowry* method (14) using bovine serum albumin as standard

Ultracentrifugation was done in a Beckman Ultracentrifuge L50 using rotor SW65 at 100000 × g/1 h

RESULTS

Effect of Milk on Heat Stable Toxin

None of the milk samples neutralized the effect of ST in infant mice

Effect of Milk on Heat Labile Toxin Tested by ELISA

Centrifuged milk apparently inhibited 50–100 % of LT when the toxin was measured by ELISA (Table 1) The inhibitory activity of the milk was partly precipitated at 50 % ammonium sulphate concentration and partly retained in the ammonium sulphate supernatant (Table 2) No correlation between the inhibitory activity and the sIgA content was observed No reduction of the inhibitory

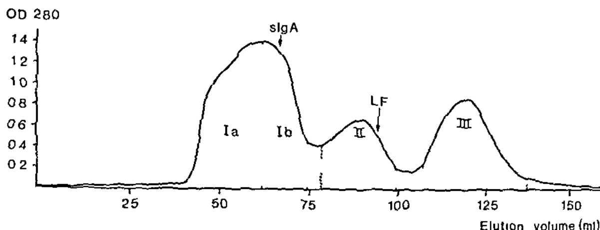
TABLE 1 *Effect of Milk on Heat Labile Toxin*

Milk no	Residual Toxin (%) in ELISA		sIgA g/l
	Undiluted milk	10 × diluted[a] milk	
1	10	30	3 2
2	20	30	15 0
3	10	–	2 5
4	20	50	1 9
5	50	80	0 5
6	0	10	4 8
7	20	20	2 8
8	[b]	30	3 8
9	10	10	11 8
10	30	40	11 0
11	10	50	1 9

a) Diluted with 0 01 M phosphate buffer pH 7 4
b) Positive toxin reaction in the absence of toxin (see text)

TABLE 2 *Effect of Milk Fractions on Heat Labile Toxin*

Milk no	Ammonium sulphate precipitate: Residual toxin (%) in ELISA, Milk fraction			Ammonium sulphate supernatant: Residual toxin (%) in ELISA, Milk fraction		
	1 g/l	0 1 g/l	sIgA content (% of total protein)	1 g/l	0 1 g/l	sIgA content (% of total protein)
1	10	40	50	<10	40	7
2	10	20	40	10	30	6
3	40	60	25	20	70	5
4	50	50	30	60	90	3
5	30	40	34	50	90	0 4
6	<10	10	14	0	0	3
7	30	30	23	0	0	12
8	50	60	71	0	70	5
9	10	10	75	0	0	0 5
10	40	70	65	80	100	0 2
11	50	50	32			



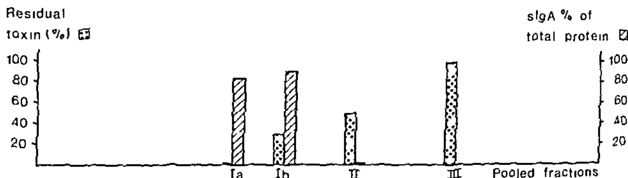


Fig 1 A Ammonium sulphate precipitate (2 ml) from milk no 6 chromatographed on an Ultrogel AcA 44 column (*70* × *2* cm) Fractions were pooled as indicated (I–III) sIgA and LF Peak positions of secretory IgA and lactoferrin
B Pooled fractions (0 1 g/l) were incubated with heat labile toxin and residual toxin measured by ELISA

activity was obtained when the ammonium sulphate supernatant of milk no 2 was passed through an anti IgA affinity column Similarly the removal by an anti IgM affinity column of IgM from the ammonium sulphate supernatant of milk no 6 resulted in no loss of the inhibitory activity Ultracentrifugation of the ammonium sulphate supernatant of milk no 6 and 9 removed 10–40% of the inhibitory activity from the solution The inhibitory activity did not prevent the binding of the second antibody (anti LT prepared in rabbit) to the toxin when the toxin was attached to the first antiserum (anti cholera toxin prepared in burro) on the ELISA plate prior to incubation with the milk fraction Preincubation of the antibody coated ELISA plate with the inhibitory fraction did not interfere with the subsequent binding of the toxin (data not shown) The inhibitory activity did not inhibit the binding of purified cholera toxin to the ELISA plate

Column chromatography of the ammonium sulphate precipitate fractions clearly indicated that the toxin inhibitory activity was eluted in front of sIgA as illustrated by the typical elution profile of milk no 6 (Fig 1) The elution patterns of IgA and lactoferrin were determined by rocket immunoelectrophoresis and their peaks are indicated (Fig 1) Similarly results of gel filtration of the ammonium sulphate supernatants indicated that the inhibitory substance had a molecular weight above 400 000 daltons since it was eluted in front of sIgA (Fig 2)

Polyacrylamide gel electrophoresis of the pooled inhibitory fractions after column chromatography of milk no 6 revealed one band in the concentrating gel and one broad band at the top of the separating gel both in areas where toxin inhibitory activity was seen (Fig 3A) SDS polyacrylamide gel electrophoresis of the same fractions under non reducing conditions indicated two main components of molecular weight 66 000 and 70 000 (Fig 3B)

Effect of Milk on LT Tested on Y1 Adrenal Cells

The milk samples did not inhibit the effect of LT on Y1 adrenal cells in culture

Fig 2 A Ammonium sulphate supernatant (2 ml) from milk no 2 chromatographed on an Ultrogel AcA 44 column (60 × 2 cm) Fractions were pooled as indicated (a–e) sIgA and LF Peak positions of secretory IgA and lactoferrin
B Pooled fractions (0 1 g/l) were incubated with heat labile toxin and residual toxin measured by ELISA

DISCUSSION

Human milk contains sIgA in high concentration as well as other well known anti microbial substances (15) which may protect breast fed infants against gastrointestinal infections

The present work demonstrated an activity in human milk which inhibited *E coli* LT measured by ELISA This inhibitory activity was partly precipitated by 50% ammonium sulphate it was not correlated with the secretory IgA content and IgA and IgM could be removed without altering the inhibitory activity No IgA IgM or IgG were detected in the most purified preparations Thus the activity is most likely of a non immunoglobulin nature

Results of gel filtration experiments indicated a

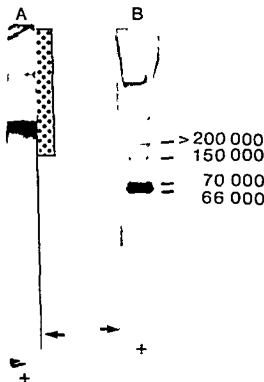


Fig 3 PAGE (tube gel) of 25 µg of peak [illegible] (see Fig 2) from milk no 6 A parallel gel was sliced and eluted as described and the area containing toxin inhibitory activity is indicated
B SDS-PAGE (slab gel) of 3 µg of peak [illegible] from milk

molecular weight of the inhibitory substance greater than 400000, while SDS-PAGE under non-reducing conditions showed two main protein bands of molecular weights 66000 and 70000 The inhibitory substance is probably an aggregate of several components, corresponding to the inhibitory activity observed in the concentrating gel and in the upper part of the separating gel after polyacrylamide gel electrophoresis (Fig 3A)

ELISA has been used in the present study to measure *E coli* LT The method is as sensitive as the Y1 adrenal cell assay (22), and the failure of the inhibitory activity to prevent the effect of LT on the cell assay suggests that the two assays are incomparable as tests for the LT inhibitory activity, which may be due to the heterogeneity among the *E coli* LT (2) The heterogenous LT may have components with activities predominant either in Y1 cell assay or in the ELISA, where the latter is more sensitive to the milk inhibitory activity

Purified cholera toxin was not inhibited by the milk inhibitory activity, indicating that the observed effect on the *E coli* LT was not due to a non-specific inhibitory activity of the ELISA assay The inhibitory activity was directed against the LT rather than the antibody-coated ELISA-plate, since preincubation of the ELISA-plate with the inhibitory fraction did not prevent the binding of LT to the ELISA-plate

[illegible] effect on of milk no 8 [illegible] ely [illegible] to the ELISA-plate In some milk samples the inhibitory activity was not reduced upon a ten fold dilution (Tables 1 and 2) possibly due to interference of other milk components Further dilution (100–1000 fold) of the milk samples always resulted in a decreased inhibitory activity

In Norway enterotoxigenic *E coli* has recently been isolated from children with diarrhoea (*Otnaess Eng & Halvorsen* unpublished observations) The toxin production by the isolated strains was low and pathogenic *E coli* strains from Norwegians may not always be identified as enterotoxigenic due to a low toxin production which requires a more sensitive assay system (11) The toxin inhibiting activity reported here may be of some practical clinical importance in Norway The nature and the possible biological relevance of the inhibitory activity is currently being investigated

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LEUCOCYTE MOBILIZATION TO SKIN LESIONS

Studies Using a Skin Chamber Technique in Healthy Volunteers

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Wandall J H Leucocyte mobilization to skin lesion Studies using a skin chamber technique in healthy volunteers Acta path microbiol scand Sect. C 88 255–261 1980

Leucocyte mobilization to a chamber covering a skin lesion was studies in healthy volunteers [111]Indium labelled blood leucocytes accumulated in the chambers indicating mobilization of leucocytes from the blood The concentration of autologous serum in the chamber medium influenced the number of leucocytes mobilized while heat inactivation of serum or the repeated use of the same serum had no effect on the counts The use of zymosan treated serum increased the mobilization by up to 53% The kinetics for the mobilization of leucocytes to chambers containing autologous serum was followed in healthy subjects The mobilization showed a uniform pattern viz a lag phase of 2–4 hours and maximal migration rates after 20–24 hours The cumulated counts were 74×10^6 leucocytes/cm^2/24 hours and 200×10^6 leucocytes/cm^2/48 hours Females and males exhibited the same kinetics and cumulated counts Chamber leucocytes were predominantly neutrophil granulocytes (85–100%) with 2 9–3 6 nuclear segments The chamber technique provides a simple method permitting quantitation of *in vivo* mobilization of leucocytes from the blood to an inflammatory lesion

Key words Local leucocyte mobilization chamber technique technical aspects

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In the living organism detrimental stimuli evoke a complex sequence of events characteristic of the inflammatory process Initially humoral changes followed by infiltration of the involved area by neutrophil granulocytes (PMN) and macrophages

The cytology of the inflammatory exudate has been extensively studied Among the methods used are observation of leucocytes in tissue sections and accumulation in rabbit ear chambers (5) In man the only widely used method has been observation of leucocytes on coverslips placed on epidermal abrasions (12) The skin window technique has proved useful in studying the cytology of the exudate in healthy subjects (12 13) and in various diseases (3 7 13 20)

However the skin window technique does not permit quantitation of the cellular migration This problem has been solved by the use of glass or perspex chambers to cover the abrasion (8 12 17) By this technique a reduced leucocyte mobilization has been demonstrated in patients with haematological diseases (9 11 14) diabetes mellitus (4) sarcoidosis (6) and psoriasis (10)

The present study was undertaken using a modification of the skin window technique to evaluate the influence of some technical variables and the mobilization of leucocytes in healthy volunteers

METHODS AND MATERIAL

Preparation of the Epidermal Abrasion

The epidermis of the volar aspect of the forearm was denuded by surgical scapels (Nos 10 11 15 and 22 Swann Morton) until the area appeared glossy and the papillary layer was visible as vividly red pin points The procedure of abrading was kept within 20–30 minutes to

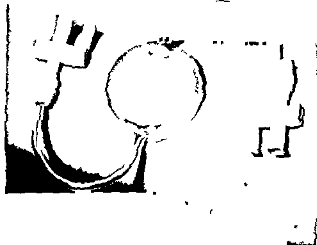


Fig 1 Chamber in position showing tubings and stopcocks

make the trauma as uniform as possible leaving an abraded area of from 1 5 to 3 5 cm^2 When the chambers were removed the abrasion was covered with semitransparent tape the lesion outlined on the tape and transferred to paper which was weighed and the area was calculated All areas were determined in duplicate

Chamber Technique

Chambers moulded from epoxy material (Araldite M HY 956 Ciba Geigy Switzerland diameter 33 mm) were fitted tubings and stopcocks (Fig 1) Sterile chambers were placed over the abrasion and fixed in position with glue (Ducocement Dupont de Nemours SA Belgium) and hypoallergenic tape Small chambers (diameter 20 mm) were used when studying various chamber media

The leucocytes were harvested from the chambers by the following procedure The chamber was drained flushed with two volumes of saline 0 15 M and refilled with fresh serum leaving an air bubble to allow movement of the fluid The serum and saline were centrifuged (150 g_{av} 10 min) the leucocytes resuspended in saline 0 15 M and counted in improved Neubauer haemocytometers the counts being made in two separate chambers The cytology of the exudate was assessed by counting 200 cells on May Grunwald Giemsa stained coverslips

Chamber Medium

Autologous serum was obtained two hours prior to the experiment filtered (0 22 um SLGS 025 OS Micropore) and stored at 4 °C In certain experiments various media were used in the chambers to study their influence upon the mobilization of leucocytes In these experiments abrasions were inflicted in the same individuals and chambers containing the different media were placed over the abrasion Chambers containing untreated autologous serum served as controls Serum was diluted with Hanks solution pH 7 3 to a final serum concentration of 50 25 and 10% heated at 56 °C for 1 hour or incubated with zymosan A (2 mg/ml Sigma Mo U S A) at 37 °C for 1 hour

Leucocyte Labelling and Protein Determination

For leucocyte labelling venous blood (50 ml) was withdrawn allowed to sediment in the presence of heparin (10 units/ml) and dextran (mol weight 250 000 Pharmacia Sweden) The erythrocytes were discarded and the leucocytes washed twice in saline 0 15 M

Indium 111 oxine was prepared as described by *Thakur et al* (18) Indium 111 (carrier free chloride solution 1 0 mCi Amersham Radiochemical Centre England) was chelated with oxine 0 35 µmoles (Merck W Germany) in ethanol and the complex formed was extracted with chloroform The chloroform was evaporated and the residue dissolved in ethanol (100 µl) to which was added saline 0 15 M (300 µl) Leucocytes were incubated with the complex for 15 min at room temperature washed twice in saline resuspended in autologous plasma and administered by a saline drip

Skin windows were inflicted simultaneously with the administration of labelled leucocytes The leucocytes were counted and the radioactivity associated with the cells after centrifugation measured

The serum concentration of alpha 1 antitrypsin and muramidase in the volunteers was measured by rocket immunoelectrophoresis (19) Rocket immunoelectrophoresis to measure the concentration of muramidase was carried out in agarose with low electroendosmosis (A 37 I Industrie Biologie Francaise) in tris barbitone buffer (pH 8 6 ionic strength 0 075) Human muramidase purified from urine served as reference

Definitions

The aim was to measure the mobilization of leucocytes and determine its kineticks The results are expressed as

1 Cumulated leucocyte migration (CLM) viz the total number of leucocytes mobilized into the chamber per cm^2 abraded epidermis
2 Leucocyte migration rate (LMR) viz the number of leucocytes accumulating in the chamber per hour per cm^2 abraded epidermis during a given period

Subjects Studied

All experiments were performed on volunteers First the influence of technical variables was investigated and secondly the mobilization into chambers containing autologous serum in 21 subjects (10 females) median age 30 years (range 20–68 years) In all experiments the chambers were emptied after 2 4 6 8 10 12 16 20 22 24 28 32 36 46 and 48 hours

None of the volunteers had a history of recent or chronic disease and all showed normal blood leucocyte and differential counts as well as serum concentration of alpha 1 antitrypsin and muramidase

At the termination of the experiment the abrasions were covered with sterile dressings left for 6 days The

48 hour medium was cultured for aerobic and anaerobic bacteria. In one instance a few aerobic organisms were found. Bleeding did not occur in any experiment and the differential counts never showed more than 1 erythrocyte per 5 leucocytes within the first 4 hours.

RESULTS

Origin of Leucocytes. Influence of Abraded Area and Emptying Procedure

Following injection of ^{111}Indium labelled autologous leucocytes radioactivity accumulated in the chambers. The radioactivity was associated with the cell pellet after centrifugation of the chamber medium. As shown in Fig. 2 the accumulation of radioactivity paralleled the appearance of leucocytes.

Increasing the abraded area from 0.1 to 4.0 cm² resulted in a corresponding increase in the number of mobilized leucocytes (Fig. 3).

To ensure that the chamber leucocytes had been sufficiently recovered, serum was drained and the chambers flushed with three separate volumes of saline 0.15 M. The leucocytes in each volume were counted and expressed as percentage of the total number of leucocytes harvested. In six experiments the serum contained 85–100% of the leucocytes, the first volume of saline 0–10%, the second volume 0–5% and the third volume no leucocytes. Therefore, the chambers were drained and flushed with two volumes of saline before being refilled with serum.

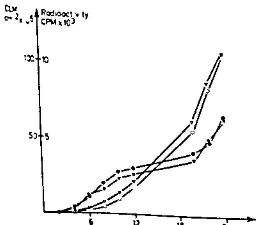


Fig. 2. Accumulation of leucocytes and radioactivity after administration of ^{111}Indium labelled leucocytes. Duplicate experiment in the same individual.

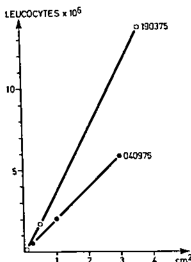


Fig. 3. Influence of size of abraded area on the mobilization of leucocytes. Experiments in two individuals.

Influence of Serum Concentration, heat Inactivation, Zymosan Treatment and Repeated Use of Serum

Serum was essential for the mobilization. Only a few leucocytes were found in chambers with Hanks solution. Raising the concentration of serum in the chamber medium increased the number of mobilized leucocytes (Table 1) which, however, remained unaffected by inactivation of serum by heat or by its repeated use (Table 2). Treatment of the serum with zymosan increased the migration by 12–53%.

Mobilization in Healthy Subjects

In 21 subjects the CLM was 50–113 × 10⁶ leucocytes /cm² (median 74 × 10⁶) at 24 hours, increasing at 36 hours to 102–261 × 10⁶ leucocytes /cm² (median 145 × 10⁶) and at 48 hours to a total of 160–413 × 10⁶ leucocytes /cm² (median 200 × 10⁶). Female subjects exhibited a CLM at 48 hours of 190 × 10⁶ leucocytes /cm², male subjects 210 × 10⁶ leucocytes /cm² ($p > 0.1$). The CLM showed no correlation to the blood leucocyte count, the PMN count, or the concentration of alpha 1 antitrypsin or muramidase.

The majority of mobilized leucocytes appeared during the first 24 hours, suggesting a slow response to the abrasion. The kinetics of this response are estimated by the LMR given in Figur 4 for 21 persons during a 48 hour period. Initially only a few leucocytes appeared in the chambers, indicating a lag phase of 2–4 hours. In the course of the subsequent hours the LMR rapidly increased, reaching a maximum at 10–12 hours. From 12 to 20 hours the LMR remained constant or slightly reduced, while at 22 to 24 hours a second increase

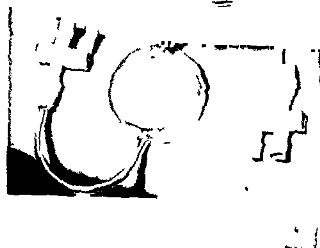


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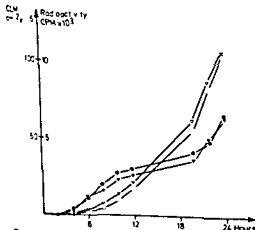


Fig 2 Accumulation of leucocytes and radioactivity after administration of ^{111}Indium labelled leucocytes Duplicate experiment in the same individual

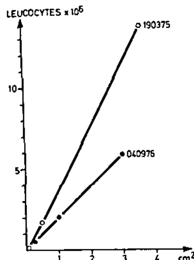


Fig 3 Influence of size of abraded area on the mobilization of leucocytes Experiments in two individuals

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TABLE 1 *Influence of Chamber Medium on the Cumulated Mobilization of Leucocytes in Three Subjects Results are Expressed as Percentage of the Mobilization Using Autologous Serum*

Chamber medium	Leucocyte mobilization	
	median	observed range
Serum 0%, Hanks sol 100%	6	1– 8
Serum 10%, Hanks' sol 90%	8	8– 16
Serum 25%, Hanks' sol 75%	15	13– 44
Serum 50% Hanks' sol 50%	37	35– 63
Heat inactivated serum	100	95–102
Zymosan treated serum	129	112–153
Previously used serum	105	76–109

TABLE 2 *Cytology of the Chamber Medium Median Value and Observed Range of 21 Experiments in 21 Healthy Volunteers*

Hours after start of skin window	Neutrophil granulocytes		Monocytes	Lymphocytes
	Percent	Mean segmentation	Percent	Percent
10–12	94 (85– 99)	3 22 (2 95–3 45)	4 (1–13 5)	1 (0–5)
24–28	97 (91–100)	3 19 (3 00–3 65)	1 5 (0– 7 0)	0 5 (0–2)
36–46	97 (95–100)	3 22 (2 87–3 60)	3 (0– 4 5)	0 (0–2)

was observed Before the experiments were terminated the leucocyte count again decreased at 46–48 hours

Cytology of the Chamber Medium

The cellular elements of the chamber fluid collected at 10–12, 24–28, and 36–46 hours were examined (Table 2) The leucocytes were predominated by PMN's with 0 2 to 1 1 nuclear segments (median 0 6) more than PMNs from venous blood Varying the accumulation period from 2 to 10 hours did not alter the cytological picture of the exudate

Macrophages were only present in small numbers In two experiments the abrasions were covered with glass coverslips after the chambers had been removed Initially PMNs accumulated on the coverslips but were replaced in 7–10 hours by macrophages

DISCUSSION

Skin windows produced by denudation of the epidermis by a surgical scalpel were introduced by *Rebuck et al* (12) and adopted by *Perillie & Finch* (11) in the glass chamber technique Scalpels with different, curved edges were preferred in the present study, as they gave uniform abrasions without bleeding and with only a few erythrocytes in the exudates *Senn et al* (15) used a high speed burr, but correction for contaminating erythrocytes was necessary, and addition of heparin induced persistent bleeding

Cantharidin applied to the skin (2) or suction (9) to produce blistering have also been used However addition of killed bacteria (2) er removal of the blister roof and basement membrane (9) proved necessary to initate a characteristic mobilization

The composition of the chamber medium is essential Maximal numbers of leucocytes were found in chambers containing undiluted serum while only a few leucocytes appeared in chambers with Hanks soltion Heat inactivation of serum did not alter the mobilization, and activation of the alternate pathway of the complement system merely induced a moderate increase Previously (8 15) it has been reported that undiluted serum is optimal for the mobilization that the essential serum factors are heat stable, non dialysable and can be isolated – on ion exchange chromatography – together with

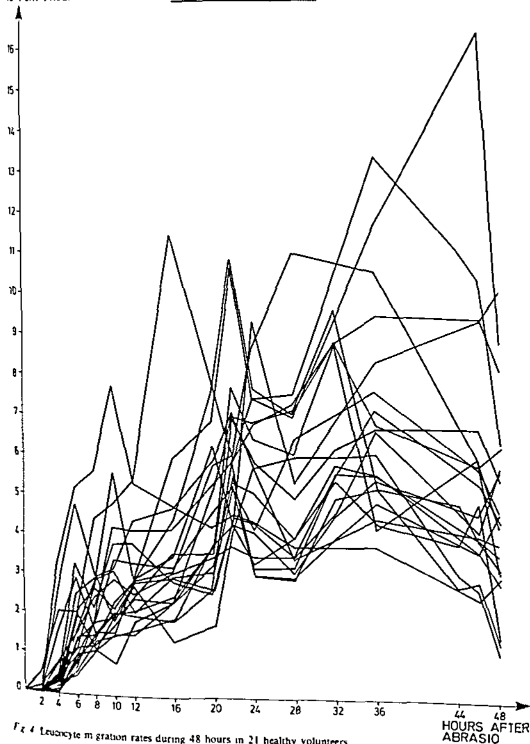


Fig 4 Leucocyte migration rates during 48 hours in 21 healthy volunteers

TABLE 1 *Influence of Chamber Medium on the Cumulated Mobilization of Leucocytes in Three Subjects Results are Expressed as Percentage of the Mobilization Using Autologous Serum*

Chamber medium	Leucocyte mobilization	
	median	observed range
Serum 0%, Hanks' sol 100%	6	1– 8
Serum 10%, Hanks' sol 90%	8	8– 16
Serum 25%, Hanks' sol 75%	15	13– 44
Serum 50%, Hanks' sol 50%	37	35– 63
Heat inactivated serum	100	95–102
Zymosan treated serum	129	112–153
Previously used serum	105	76–109

TABLE 2 *Cytology of the Chamber Medium Median Value and Observed Range of 21 Experiments in 21 Healthy Volunteers*

Hours after start of skin window	Neutrophil granulocytes		Monocytes	Lymphocytes
	Percent	Mean segmentation	Percent	Percent
10–12	94 (85– 99)	3 22 (2 95–3 45)	4 (1–13 5)	1 (0–5)
24–28	97 (91–100)	3 19 (3 00–3 65)	1 5 (0– 7 0)	0 5 (0–2)
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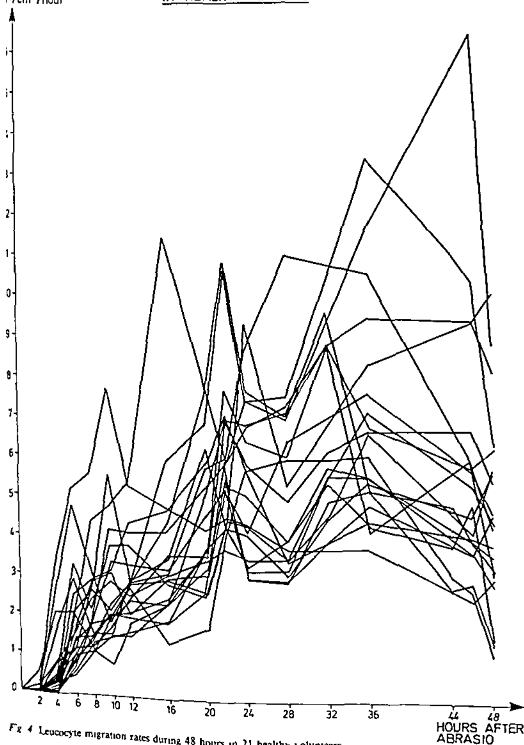


Fig 4 Leucocyte migration rates during 48 hours in 21 healthy volunteers

the gamma globulin fraction (15) *In vitro* activation of the complement system generates leucotactic factors (16) In the skin window complement factors may be of less importance, as heat inactivation of the serum did not reduce the number of cells mobilized

A lag phase was observed in the present study as it has been in others (2, 8, 9, 11) Perhaps, it merely reflects the distance the leucocytes have to travel to reach the abrasion This concept is supported by the finding of *Senn et al* (15) of a brief lag phase in skin windows contaminated with erythrocytes, as a sign of deep abrasions The subsequent increase in migration suggest an interaction between the leucocytes arriving first and the serum Possibly, the alteration takes place in the lesion itself, seeing that repeated use of the serum did not increase the mobilization Mobilization of leucocytes may be initiated by PMN lysates (8), and it has been suggested that an interaction with membrane bound esterases initiates migration (1)

Leucocyte mobilization in healthy persons has been described previously in studies using different methods of producing skin windows and different media (7, 9, 11, 14) The values found have been discrepant, but in keeping with differences in the techniques Undiluted serum was used as a medium by *Senn* (14) who found a cumulated mobilization of 69 $\times$ 10^6 leucocytes/cm^2/24 hours compared with 74 $\times$ 10^6leucocytes /cm^2/24 hours in the present study *Senn* (14, 15) described a »peak type« mobilization in male subjects and an »up slope« mobilization in females No indication of such different mobilization types was found in the present study or in other studies in which scalpels (4, 11) or suction (9) have been used to produce the skin window The explanation of these differences is possibly technical as *Senn et al* (15) reported different types of mobilization to simultaneous chambers in the same individuals

In all studies using the chamber technique the mobilized leucocytes have been predominantly PMNs Macrophage predominance is a regular finding at 6–10 hours when using the original skin window technique (12 13) The occurrence of a macrophage phase when glass coverslips are placed on the abrasion may reflect a foreign body reaction or displacement of the PMNs from the glass surface

The skin window represents the only method applicable in man for studying the inflammatory response *in vivo* By the chamber technique the mobilization of leucocytes from the blood to the inflammatory lesion can be quantitated Furthermore the total number of cells harvested from the chamber permits supplementary studies of leucocyte function *in vitro*

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ANTIGENS AND ANTIBODIES IN *PLASMODIUM FALCIPARUM* MALARIA STUDIED BY IMMUNOELECTROPHORETIC METHODS

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Jepsen S & Axelsen N H Antigens and antibodies in *Plasmodium falciparum* malaria studied by immunoelectrophoretic methods Acta path microbiol scand Sect C 88 263–270 1980

Soluble plasma antigens in 53 Liberian patients with *P falciparum* malaria were analyzed in double diffusion using plasma from 27 immune adults One immune plasma reacted with 17% of the plasma samples and was used in subsequent quantitative immunoelectrophoretic (IE) studies as antiserum By fused rocket IE 2 antigens were found in samples with parasitaemia (P) above 0 6% and above 4 5% P the antigens had grossly increasing concentration In 22 samples of 0 6% P or more 7 antigens were demonstrated by crossed IE Two electrophoretically heterogeneous antigens Ag 1 and Ag 2 were found most frequently Ag 1 was partly heat stable and was in some samples amphiphilic as shown by charge shift crossed IE Ag 1 and Ag 2 did not contain integral erythrocyte membrane proteins and Ag 1 is suggested to be a structural protein of *P falciparum* Twenty six immune plasma samples were screened for antibodies against 4 antigens in crossed IE with intermediate gel It is suggested that an index for protective immunity may be obtained by summation of semiquantitative titers obtained by crossed IE with intermediate gel using an antigen pool representing e g 20–30 soluble antigens

Key words *Plasmodium falciparum* malaria soluble antigens precipitating antibodies

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McGregor et al (1968) provided evidence by using double diffusion in gel that antibodies from immune adults react with soluble antigens present in blood from children with *P falciparum* infection At least 30 different antigens of which 20 are heat stable (S antigens stable at 100 °C for 5 min) have been demonstrated by matching very large series of patients sera and immune sera (*Wilson et al* 1969 *Wilson et al* 1975 *McGregor & Wilson* 1971) None of these antigens have been isolated but some antigens have been partially characterized by gelfiltration sucrose gradient ultracentrifugation heat succeptibility and resistence to degradation by various enzymes (*Wilson et al* 1969 *Wilson et al* 1973 *Wilson* 1974) Autoradiographic studies of extracts of parasitized red cells maintained in vitro in presence of radioactive aminoacids showed that S antigens were not labelled in contrast to some L-antigens (L antigens destroyed at 56 °C for 30 -

… 1974) These results strongly support the hypotheses that soluble antigens from *P falciparum* are released during the infection and may play an important role in the host parasite relationship

In the present study we have applied quantitative IE methods to the study of soluble malaria antigens and their corresponding antibodies The results are in accordance with some of the earlier observations

MATERIALS AND METHODS

Antigen Material

Seventy patients attending the outpatients clinic at the Steven A Tolbert Memorial Hospital Buchanan, Grand Bassa county, Liberia and the LAMCO Hospital, Yekepa Nimba county, Liberia during the month of June *1977* for treatment of malaria were prior to chemotherapy bled by venepuncture in sterile EDTA tubes (Nunc Denmark) Case selection was conditional upon fever and the presence of *P falciparum* in thick blood films prepared from fingerprick Thin blood films for determination of parasitaemia were made from the EDTA blood samples and stained with 4% Giemsa The results are reported as percent parasitaemia, that is, the ratio of infected erythrocytes to the total number of erythrocytes counted In each specimen 3000 to 5000 erythrocytes were examined by 2 persons at 1000 × under oil Seventeen patients were excluded from the investigation for reasons such as infection with more than 1 malaria species (often *P malariae*) or evidence of concomitant infections

EDTA blood samples were spun at 2000 × *g* for 3 minutes and to the plasma was added Aprotinin (Trasylol® Bayer W Germany) and NaN_3 to a concentration of 100 K I E/ml and 15 mM respectively storage was at −20 °C

Antisera

Immune sera Four aliquots of acid citrate dextrose plasma were obtained from outdated blood from the blood banks of the above mentioned hospitals Samples of 10 ml EDTA plasma were obtained from 23 apparently healthy African adults After addition of Aprotinin and NaN_3 as described above the samples were stored at −20 °C Serum was prepared from the samples by recalcification

Plasma was used in double diffusion in gel whereas serum was used in fused rocket immunoelectrophoresis crossed immunoelectrophoreses and charge shift crossed immunoelectrophoreses

Rabbit antiserum Rabbit immunoglobulin precipitating the intrinsic (integral) membrane proteins of human erythrocytes was purchased from Dako Immunoglobulins Ltd Copenhagen Denmark (Code A 104 lot 047)

Double Diffusion in gel (Ouchterlony & Nilsson *1978*) was performed in 1% agarose gel (Litex Glostrup Denmark type HSA) in Tris-barbital buffer pH 8 6 ionic strength 0 02 on glass plates 10 × 10 cm The wells 4 mm in diameter contained 10 µl of the reagents and were 7 mm apart (centre to centre) Diffusion proceeded for 24 hours at 20 °C and reading was performed both in the wet state and after staining with Coomassie brilliant blue R 250

Fused Rocket Immunoelectrophoresis (Svendsen *1973*) was performed on glass plates 10 × 20 cm in 1% agarose gel using the same buffer and gel as in the double diffusion test 10 µl samples were applied in wells punched in a broad gel without antiserum Electrophoresis proceeded at 2 V cm^{-1} for 18 hours at 12 °C The plates were washed, pressed and stained with Coomassie brilliant blue R-250

Crossed Immunoelectrophoresis with Intermediate Gel (Axelsen *1973*) was performed on glass plates 7 × 5 cm in 1% agarose gel using the buffer and gel described above In first dimension 20 µl antigen (plasma) was run with a potential gradient of 10 V cm^{-1} for 30 minutes at 12 °C The second dimension gel contained 400 µl immune serum No 1, and 2 V cm^{-1} was applied for 18 hours at 12 °C In different experiments the intermediate gel contained 150 µl immune serum from different persons 100 µl rabbit antierythrocyte membrane Ig or 0 1 M NaCl (control plates) The plates were washed pressed and stained with Coomassie brilliant blue R-250

Charge-shift Crossed Immunoelectrophoresis (Bhakdi et al *1977*) was performed as crossed immunoelectrophoresis as described above First dimension electrophoresis was performed in 3 different gels containing 0 5% Triton X-100 (control plate) 0 5% Triton X-100 + 0 2% desoxycholate (DOC) and 0 5% Triton X-100 + 0 0125% N cetyl-N N N trimethylammoniumbromid (CTAB), respectively Second dimension gels contained in all 3 experiments (400 µl) immune serum No 1 and 0 5% Triton X-100 The first dimension electrophoresis was run until a hemoglobin marker had migrated 20 mm measured from the anodic edge of the application basin to the forefront of the hemoglobin band An amphiphilic protein will in this set up change its migration distance at least 5 mm in both DOC and CTAB

RESULTS

Parasitaemia in the 53 antigen (plasma) samples varied from 0 2% to 24 5%

Double Diffusion-in-gel

The diffusion was carried out with all 53 antigen (plasma) samples against the 27 plasma samples from immune adults

Thirty (57%) of the 53 antigen samples and 19 (70%) of the 27 antiserum samples formed precipitate at least with one antiserum and antigen sample respectively One antiserum (No 1) was among the 5 best antisera reacting with 17% of the antigen samples This antiserum, which was available in appropriate quantities, was selected for further studies by quantitative IE (see below)

Of the 23 non-reacting antigen samples 13 originated from patients with parasitaemia below 0 6% All but one of the plasma samples from patients with parasitaemia of more than 4% reacted with one or more antisera The exception was a sample from a child with a parasitaemia of 6 5% Increasing parasitaemia was positively correlated to

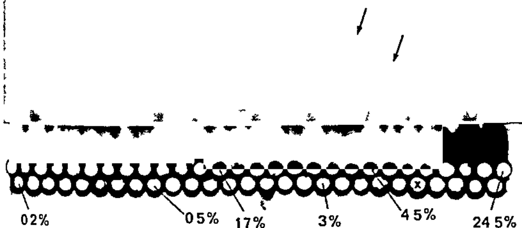


Fig 1 Fused rocket IE of 53 plasma samples from *P falciparum* infected patients against immune serum No 1 The plasma samples were arranged in order of increasing parasitaemia from left to right First and last well empty and sample No 6 (from left) excluded in control experiments for reasons mentioned under materials and methods

Two antigens were detected above 0 5% parasitaemia and they showed a grossly parallel variation above 4 5 % parasitaemia

Technical 10 μl plasma samples 1600 μl immune serum anode at the top electrophoresis at 2 V cm⁻¹ for 18 h staining Coomassie brilliant blue R 250

reaction with an increasing number of antisera Thus 11 samples with parasitaemia above 3% precipitated with 4 or more antisera

Fused Rocket Immunoelectrophoresis

Plasma samples were arranged in order of increasing parasitaemia and electrophorized against immune serum No 1 Fig 1 shows that no antigens were found in the range 0 2–0 5% parasitaemia In the range 0 5–1 7% 1 antigen was demonstrated in 4/6 of the samples In the range 1 7–3% parasitaemia 2 unrelated antigens with different precipitate morphology were found in 9 of the 12 samples and none in 3 samples In the range 3–24 5% parasitaemia the 2 antigens could constantly be demonstrated in all samples but one (× in Fig 1) and above 4 5% P the antigens generally showed an increase in concentration

The same experiment as shown in Fig 1 was performed with cathode at the top in order to detect antigens migrating towards the cathode No such antigens could be demonstrated

Crossed Immunoelectrophoresis

The nineteen antigen samples with highest parasitaemia (3 5–24 5%) and 6 samples in the low range (0 2–3%) were selected for crossed immunoelectrophoresis in order to investigate whether more antigens than the 2 revealed in Fig 1 could be detected and to study the electrophoretic mobility and profile of the antigens As antiserum was used No 1

The twenty five crossed immunoelectrophoreses plates were compared and as judged from the characteristics of the precipitates i e morphology electrophoretic profile and position a total of 6 antigens were found Fig 2 is a composite drawing of the findings and a typical appearance of precipitate No 1 and No 2 is seen in Fig 3 The findings are summarized in Table 1 Antigen No 1 displayed in most instances 3 peaks without spurring 1a was in a few instances split in 2 peaks Peak 1c had in all samples an irregular tailing and often a double contour indicating that it was electrophoretically less homogeneous than 1a and 1b Peak 1b was always larger than 1a and 1c and was very slender distinct and pointed The electrophoretic mobility of antigen 1b was equal to that of human prealbumin Antigen No 2 appeared in most instances as a double peak and the precipitate was thin but distinct and showed no interference with precipitate No 1

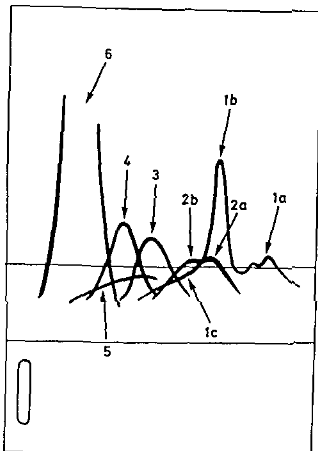
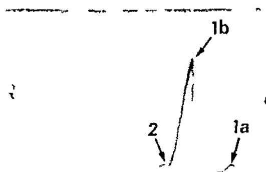


Fig 2 Composite drawing of the six antigens found by testing plasma from 25 patients against immune serum No 1 in crossed IE



Six antigen samples containing Ag 1 and Ag 2 were tested in crossed immunoelectrophoresis with rabbit anti human erythrocyte membrane antibodies in the intermediate gel and antiserum No 1 in the upper (reference) gel The rabbit antibodies did neither precipitate antigen No 1 nor No 2

Heat Stability

Seven antigen samples were heated at 100 °C for 5 min and tested in crossed IE against antiserum No 1 Antigen 1b was stable in all tests and 1a and 1c were unstable in a few experiments Antigen 2a and 2b were destroyed at 100 °C but resisted heating at 56 °C for 30 min The findings are summarized in Table 1

Charge Shift Crossed IE

Seven plasma samples with suitable concentrations of antigen 1 and 2 were selected for this test In 2 samples antigen 1 displayed a complete charge shift of 5 mm with both DOC and CTAB as seen in Fig 4 In the other 5 samples the charge shift of antigen 1 was incomplete i e less than a 5 mm shift or only a shift with CTAB Antigen No 2 showed no charge shift The results are summarized in Table 1

Occurrence of Antibodies in 26 Immune Sera

Twenty six sera were tested for the presence of antibodies against antigen 1a b c and 2a b in crossed IE with intermediate gel Plasma sample No 5 was used as antigen and antiserum No 1 as reference in the upper gel Antibodies against antigen No 1 in varying titers were found in 13 (50%) of the immune sera (Table 2) Nine (35%) immune sera contained antibodies against antigen No 2 but in relatively low titers Two additional antigens x and y were found in these experiments As judged from precipitate morphology profile and electrophoretic position Ag x most likely is identical to Ag 6 whereas Ag y with an electrophoretic mobility higher than Ag 1a was not seen before It appears from Table 2 that anti y is as frequent as anti 1 in the immune sera studied but there was no correlation between the occurrence of the 2 antibody

Fig 3 Crossed IE with intermediate gel (containing 250 µl 0 1 M NaCl) of a patient s plasma against immune serum No 1 Note the very slender peak of antigen No 1b

Technical 20 µl patient s plasma 400 µl immuneserum 1st dim electrophoresis at 10 V cm $^{-1}$ for 30 min 2nd dim electrophoresis at 2 V cm $^{-1}$ for 18 h staining Coomassie brilliant blue R 250

TABLE I *Number Heat stabil ty and Charge Shift of Antigens Demonstrated by Crossed Immunoelectroph resis in Plasma of 25 Patients with P* falciparum *Infection*

Sample No	Parasitaemia (%)	Antigen No									Heat stability[a] Ag No					Charge shift[b] Ag No	
		1a	1b	1c	2a	2b	3	4	5	6	1a	1b	1c	2a	2b	1	2
1	24 5	+	+	+	+	+					+	+	+	−	−	(+)	−
2	13	+	+	+	+	+					+	+	+	−	−	+	−
3	12	+	+	+	+	+					+	+	+	−	−	(+)	−
4	11 5		+														
5	10	+	+	+	+	+					+	+	+	−	−	(+)	−
6	10	+	+														
7	9	+	+	+	+		+	+									
8	7 5	+	+	+	+						+	+	+	−		(+)	−
9	6 5																
10	5 5									+							
11	5		+						+								
12	5		+	+													
13	5	+	+	+	+	+	+	+			−	+	−	−	−	(+)	−
14	4 5		+	+													
15	4 5		+	+													
16	4		+	+	+							+	−	−		+	−
17	4																
18	4		+	+	+	+											
19	3 5																
20	3	+	+	+				+									
21	2		+	+	+	+											
22	0 6							+									
23	0 3																
24	0 2																
25	0 2																

[a] 7 samples heated at 100 °C for 5 min + (stable) − (unstable)
[b] 7 samples tested + (charge shift with both DOC and CTAB) (+) (charge shift only with CTAB) − (no charge shift)

specificities Fig 5 shows an experiment in which anti 1 and anti y were demonstrated in immune serum No 13

DISCUSSION

Our results confirm some of the results previously reported concerning soluble antigens and their corresponding antibodies in *P falciparum* malaria (*McGregor et al* 1968 *Wilson et al* 1969 *McGregor & Wilson* 1971 *Wilson et al* 1975) In spite of our relatively small clinical material it was possible to demonstrate 7 different antigens by analys s of 25 selected

much larger materials using a less sensitive technique

The number of parasitized erythrocytes per µl blood is a more relevant measure of parasite load than the parasitaemia but was not obtainable *in this* study However fused rocket IE showed that the antigen concentration grossly increases with increasing parasitaemia above 4 5%. This finding *is in* accordance with the observations using double diffusion technique Therefore *by* using more sensitive techniques it may be possible to *demon*strate soluble antigens in all cases of acute *P falciparum* malaria. We have no *explanation* for the absence of antigens in the sample marked X in Fig 1

Antigens Nos 1 and 2 were the most frequently detected antigens and displayed electrophoretic heterogeneity in all *samples* Antigen No 1b gave a

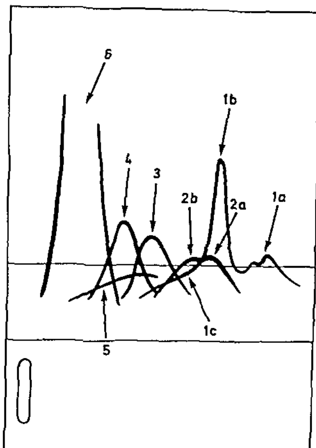
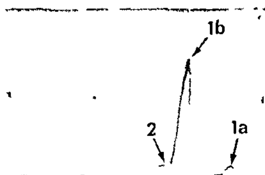


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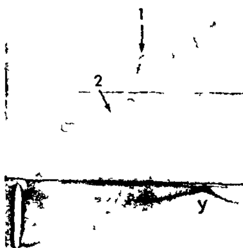


Fig 5 Crossed IE with intermediate gel containing anti 1 (precipitate extending to 1st dim gel) anti 2(deflection of precipitate) and anti Y antibodies Note the fast mobility of antigen Y

Technical 20 µl patient s plasma (No 5) Intermediate gel contained 150 µl immuneserum (No 13) Upper reference gel contained 400 µl immuneserum No 1 1st dim electrophoresis at 10 V cm ¹ for 30 *min* 2nd *dim* electrophoresis at 2 V cm ¹ for 18 h Staining Coomassie brilliant blue R 250

1974) to be of host (erythrocyte?) origin However Ag 1 did not react with rabbit anti human erythrocyte membrane *proteins and* Ag 1 therefore does not contain any of the known amphiphilic proteins of the human erythrocyte *membrane* We find it an interesting possibility that Ag 1 is a *P falciparum* membrane protein released during the malaria attacks but more direct evidence is needed to confirm this hypothesis Anti Ag 1 antibodies are strongly precipitating indicating that they most probably not are allo or autoantibodies The use of EDTA may have facilitated the solubilization of Ag 1 but it is likely that Ag 1 is sufficiently water soluble to be a circulating antigen since it easily appeared *in* crossed IE using agarose gels with no

... ore belongs to the R-class (*McGregor et al* 1968) and it showed no reaction with rabbit anti human-erythrocyte membrane antiserum

Due to the high frequency of Ag 1 and Ag 2 in the patients a series of 26 immune sera were screened for antibodies against these two antigens The antibody findings summarized in Table 2 are in accordance with earlier findings showing that antibodies in immune persons represent a broad spectrum of specificities occurring in unexplainable combinations at the present stage of knowledge (*McGregor & Wilson* 1971 *Wilson et al* 1976) The antibody titers were semiquantified and also the titers varied very much The screening furthermore revealed that the antigen sample in addition to Ag 1 and Ag 2 contained at least 2 additional antigens x and y and we suggest that Ag x is identical to Ag 6 The 26 sera were therefore in fact screened for antibodies against 4 antigens

The demonstrated antibody specificities may contribute protective immunity but if so other specificities must also be protective since no antibodies were demonstrated in 5 of the immune persons (Nos 5 16 24 25 26 Table 2) We therefore suggest that the protective immunity to *P falciparum* might be measured as the sum of semiquantitative antibody titers found by screening sera in crossed IE with intermediate gel This would require as reference antigen a pool of selected sera from malaria patients To be able to detect antibodies in low titers an antiserum pool reacting with these antigens should be used as reference antiserum When tested in this way one serum only needs one analysis for detection identification and semiquantitative titration of all antibody specificities (*Axelsen* 1973) and the inter individual differences in antibody response is minimized (*Axelsen* 1976) Selection of suited reference antigens and antisera obviously requires screening of very large clinical materials or production of antigens by *in vitro* cultivation of *P falciparum* and immunization of rabbits for production of reference antiserum

greatly acknowledged The study was done under ...

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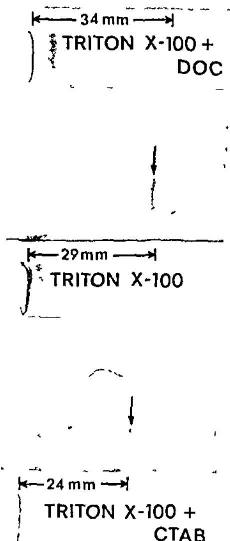


Fig 4 Charge shift of antigen No 1b (arrows) in presence of DOC (desoxycholate) and CTAB (N cetyl-N N N trimethylammoniumbromid)

Technical 20 µl patient's plasma first dimension gel contained detergents as indicated Second dimension gel contained 400 µl immune serum No 1 and 0 5 % Triton X 100 First dimension electrophoresis was run until a hemoglobin marker had migrated 20 mm measured from the anodic edge of the application basin to the forefront of the hemoglobin band Second dimension electrophoresis at 2 V cm⁻¹ for 18 h staining Coomassie brilliant blue R 250

TABLE 2 *Antibodies in 26 Immune Sera Demonstrated by Crossed IE with Intermediate Gel Using Antigen Sample No 5 and Immune Serum No 1 as Reference*

Immune serum No	Antibodies against antigen			
	No 1	No 2	x	y
2	++	–	+++	–
3	+++	–	+++	–
4	++	–	+++	–
5	–	–	–	–
6	–	–	–	+++
7	++	–	–	+++
8	+	+	–	–
9	++	+	–	+++
10	+	+	–	+++
11	++	–	–	+++
12	+++	–	–	+++
13	++	+	–	+++
14	+	–	–	–
15	–	+	–	–
16	–	–	–	–
17	–	+	–	+++
18	–	–	–	+++
19	–	++	–	+++
20	–	–	–	+++
21	–	+	–	+++
22	–	++	–	+++
23	++	–	+++	–
24	–	–	–	–
25	–	–	–	–
26	–	–	–	–
27	++	–	–	–

+++ a high titer (precipitate located close to or reaching the 1st dimension gel (*Axelsen* 1973))
++ a medium titer (between +++ and +)
+ a low titer (reference precipitate deflected or extended into the intermediate gel (*Axelsen* 1973))

very characteristic slender, distinct and pointed precipitate, and Ag 1c the »tail« of Ag-1 was irregular and often double-contoured indicating that Ag-1 possibly was of amphiphilic nature (*Bjerrum* 1977) This suggestion was supported by the finding that Ag-1 in two samples showed charge shift with both DOC and CTAB In 5 other samples Ag 1 only showed charge shift with CTAB These observations indicated that Ag-1 might represent a more or less solubilized integral membrane protein (antigen) from *P falciparum* or possibly a complex of a *P falciparum* antigen and an erythrocyte membrane protein Ag-1 was heat stable and therefore belongs to the S-class of antigens demonstrable in *P falciparum* malaria suggested (*Wilson*

cta path microbiol scand Sect C 88 271–274 1980

THE GROUP A STREPTOCOCCAL RECEPTOR FOR HUMAN IgA BINDS IgA VIA THE Fc-FRAGMENT

CLAES SCHALEN

Department of Medical Microbiology University of Lund Sweden

Schalen C The group A streptococcal receptor for human IgA binds IgA via the Fc fragment Acta path microbiol scand Sect C 88 271–274 1980

Eight freshly isolated type M4 strains of group A streptococci were found to bind between 60 and 80% of 2 5 µg radiolabelled IgA myeloma protein in a standard test system while a reference type 4 strain bound only 20% Commercial human IgG or IgG1 myeloma protein did not inhibit the binding of IgA by the reference type 4 strain or one of the freshly isolated type 4 strains whereas inhibition was obtained by purified polyclonal IgA and IgA1 myeloma protein Fc fragments of purified IgA1 myeloma protein obtained by digestion with gonococcal protease inhibited binding of radiolabelled IgA1 while the Fab fragments had no inhibitory effect

Key words Streptococci human IgG human IgA Fab fragment Fc fragment

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Accepted as submitted 20 v 80

Many group A C and G streptococci have receptors for human IgG (1 3 4 6 7 10) while IgA receptors have been demonstrated in only a few strains of group A streptococci (5 10) *Christensen & Oxelius* (5) reported that the binding of IgA myeloma proteins to group A streptococci type 4 was not significantly inhibited by IgG and *vice versa* Recently separate receptors for human IgG and IgA were demonstrated in alkaline extract of type 4 group A streptococci (12)

The interaction between IgG and the corresponding streptococcal receptors has been localized to the Fc fragment of IgG (1 3 6) The present paper concerns the interaction between Fab and Fc fragments of IgA and the streptococcal IgA receptors A recently described method for cleavage of IgA by gonococcal IgA protease was used to produce these IgA fragments (11)

MATERIALS AND METHODS

Bacterial Strains and Preparations

The group A streptococcal reference strains M4 (SS241) and M15 (EF 1949) and 8 freshly isolated type M4 strains were used in IgA binding experiments Standard suspensions of the strains were prepared as described by *Christensen & Oxelius* (4)

The gonococcal strain used for cleavage of IgA has been subcultivated five times a week for several years in our laboratory (strain 3K)

Immunoglobulin Preparations

Human purified polyclonal IgA and one IgG1 and one IgA1 kappa-chain M-component were kindly supplied by Dr *A Grubb* Malmo General Hospital Malmo Sweden [illegible]

sed [illegible]

bel [illegible]

receptor and were also used in unlabelled state for inhibition studies

Digestion of Monoclonal Human IgA1 by Gonococcal Protease

The gonococcal strain 3K was grown overnight at 37 °C in 6% CO_2 after heavy inoculation of two haematin agar plates (9 cm diameter) The colonies were harvested and suspended in 5 ml of IgA myeloma protein 10 *mg/ml* in phosphate buffered saline (PBS

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THE GROUP A STREPTOCOCCAL RECEPTOR FOR HUMAN IgA BINDS IgA VIA THE Fc-FRAGMENT

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Immunoglobulin Preparations

Human purified polyclonal IgA and one IgG1 and one IgA1 kappa chain M-component were kindly supplied by Dr *A Grubb* Malmo General Hospital Malmo Sweden Human commercial IgG was purcha sed from AB Kabi Sweden (batch No 70972)

The IgA1 and IgG1 myeloma proteins were radiola belled with ^{125}I (9) for detection of streptococcal IgA receptor and were also used in unlabelled state for inhibition studies

Digestion of Monoclonal Human IgA1 by Gonococcal Protease

The gonococcal strain 3K was grown overnight at 37 °C in 6% CO_2 after heavy inoculation of two haematin agar plates (9 cm diameter) The colonies were harvested and suspended in 5 ml of IgA myeloma protein 10 mg/ml in phosphate buffered saline (PBS

0 03 M phosphate 0 12 M NaCl pH 7 2) The mixture was incubated at 37 °C in rotating tubes for 17–18 hours centrifuged at 10000 *g* and the supernatant sucked off and sterile filtered The digested IgA myeloma protein was analyzed by immunoelectrophoresis (13)

Preparatory Electrophoresis

Preparatory electrophoresis was performed in a 4 mm layer of 1% agarose (Miles Ltd England) (in 0 075 M barbital buffer pH 8 6 containing 2 mM calcium lactate) poured over a 205 × 110 mm glass plate (2) The crude digested IgA preparation was applied in a 6 mm broad channel 190 mm long cut in the agarose gel 75 mm from the projected anode A voltage of 150 V was applied for 2 hours after which the gel was cut into 5 mm wide slices After freezing the pieces overnight at −80 °C followed by thawing and centrifugation at 3000 *g* protein containing supernatants were harvested

Separation of Digested IgA M Component by Gel-Filtration

The digested IgA myeloma protein was separated on a Sephadex G200 column (2 5 × 90 cm filtration in PBS at a rate of 18 ml/hour 3 ml fractions)

Rabbit Antisera

Antiserum to IgA and antiserum to kappa chain were purchased from DAKO Copenhagen Denmark

Protein Determinations

Protein concentrations were measured with a modification of *Folin* s method (8)

Determination of the Uptake of Radiolabelled IgA1 and IgG1 by Streptococci Inhibition Experiments with Immunoglobulins and IgA fragments

The binding of radiolabelled IgA1 and IgG1 myeloma protein to the streptococci was measured as described by *Christensen & Oxelius* (4 5) with slight modifications An excess of streptococcal Ig receptors is allowed to react with a constant amount of radiolabelled Ig PBS 100 µl containing 0 1% (vol/vol) Tween 20 (in order to minimize unspecific protein adherence) was mixed with 200 µl of the streptococcal standard suspension and 50 µl radiolabelled IgA1 or IgG1 (2 5 µg) After 30–45 min at 22 °C 2 ml PBS containing 0 1 % Tween 20 was added the tubes centrifuged at 3000 *g* and the supernatant sucked off The radioactivity in the pellet was expressed as a percentage of the total amount of radioactivity added In the inhibition experiments with different immunoglobulin preparations the preparation to be investigated was first mixed with the streptococci and PBS containing 0 1 % Tween 20 and left at 22 °C for 2–5 min After the addition of 50 µl radiolabelled IgA1 (2 5 µg) the binding of IgA1 to the streptococci was tested as described above

RESULTS

Binding of Radiolabelled IgA1 and IgG1 by Some Group A Streptococci

All 8 freshly isolated type M4 strains investigated bound 60 to 80% of the ^{125}I labelled IgA1 myeloma protein In contrast the reference strain of type M4 SS241 (which had been kept and subcultivated in the laboratory) showed an uptake of only 20% The uptake of type 15 group A streptococci was below 5% In the following experiments two strains were used the reference strain of type M4 and one freshly isolated M4 strain (595)

In order to investigate the specificity of the IgA binding various unlabelled immunoglobulin prepa

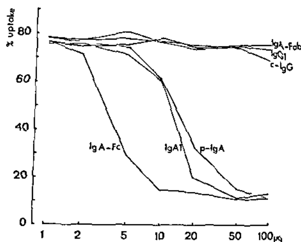


Fig 1 The effect of addition of various unlabelled immunoglobulin preparations on the binding of radiolabelled IgA1 myeloma protein to type M4 streptococci strain 595 Abscissa µg of unlabelled immunoglobulin added Ordinate uptake of radiolabelled IgA1 myeloma protein in % of 2 5 µg added IgA Fab Fab fragments of IgA1 IgA Fc Fc fragments of IgA1 IgA1 IgA1 myeloma protein IgG1 IgG1 myeloma protein c IgG commercial human IgG and p IgA polyclonal IgA

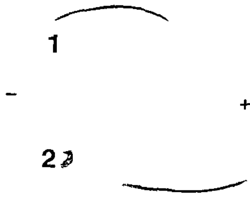


Fig 2 Immunoelectrophoresis of digested (gonococcal protease) and undigested IgA1 myeloma protein Well 1 application well for undigested IgA1 Well 2 application well for digested IgA1 Middle trough anti kappa serum Upper and lower troughs anti IgA serum + indicates anode

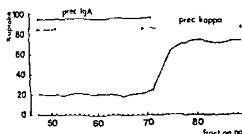


Fig 3 The effect of addition of unlabelled fractions obtained from gel filtration of gonococcal protease digested IgA1 myeloma protein on the binding of radiolabelled IgA1 myeloma protein to type M4 streptococci strain 595 Abscissa indicates fraction No Ordinate uptake of radiolabelled IgA1 myeloma protein in % of 2 5 µg added Unbroken line indicates fractions precipitated by anti IgA serum Broken line fractions precipitated by anti kappa serum

rations were tested for their capacity to inhibit the binding of radiolabelled IgA1 to the streptococci using 1–100 µg (200 µl volumes) of the unlabelled compounds (Fig 1) Increasing amounts of polyclonal IgA and IgA1 M component suppressed the uptake of IgA1 on strains 595 and SS241 On the other hand no inhibition was found on addition of IgG1 *M component or commercial human IgG*

The uptake of ^{125}I labelled IgG1 myeloma protein by the 8 freshly isolated type M4 strains was between 7 and 45 (mean 26) % of 2 5 µg added as compared to an uptake of 11 % shown by the M4 reference strain There was no correlation between uptake of IgG1 and IgA1 in the single strain

Test of the Capacity of IgA1 Fragments to Inhibit the Binding of IgA1 to Type 4 Group A Streptococci

Digested IgA1 separated by gel filtration IgA1 myeloma protein 50 mg was digested with gonococcal protease Fig 2 shows an immunoelectrophoresis of digested and undigested IgA1 M component involving anti IgA and anti kappa serum The digested IgA showed an anodal displacement of the precipitate *obtained with anti IgA* as compared with undigested IgA while the precipitate given by anti kappa serum had moved slightly cathodally The digested protein was filtered on a Sephadex G200 column and 500 µl of each fraction was examined for capacity to inhibit the binding of radiolabelled monoclonal IgA1 by type 4/595 (Fig 3) and SS241 Fractions 41–71 precipitated with anti IgA serum in double diffusion in gel Fractions 45–51 and 69–89 precipitated with anti kappa serum Only the fractions precipitated by anti IgA serum inhibited the binding af radiolabelled IgA1 to the two type 4 strains (Fig 3)

From the results of molecular weight determinations on calibrated Sephadex G200 column (performed by Dr *A Grubb* Malmo) it was concluded that the fractions 53–67 were aggregated Fc fragments while fractions 73–89 *represented* Fab fragments (Fig 3) The Fab and Fc fragments were pooled separately A decrease *in binding of* radiolabelled IgA1 *myeloma protein to* strains 595 (Fig 1) and SS241 was found on addition of increasing amounts of Fc *fragments* while similar amounts of Fab fragments had no effect

Digested IgA1 separated by preparatory electrophoresis The digested IgA1 myeloma protein 10 mg

TABLE 1 *Inhibition Studies on the Uptake of Radiolabelled IgA Myeloma Protein on Type 4 Group A Streptococci (Strains 595 and SS241) Using Fractions Obtained from Preparatory Electrophoresis of Digested IgA Myeloma Protein*

Fraction no	Precipitated in double diffusion in gel by		Addition of the fraction effect on uptake of radiolabelled IgA (in %) by	
	anti IgA serum	anti kappa serum	strain SS241	strain 595
2	–	–	17	73
3	–	–	17	73
4	–	–	17	75
5	–	+	16	73
6	+	+	3	16
7	+	+	2	15
8	+	–	5	37
9	+	–	4	48
10		–	16	74
11	–	–	16	73
12	–	–	17	73

was subjected to preparatory electrophoresis All fractions (200 μl) that precipitated with anti IgA inhibited as also the anodal fractions 8 and 9 (Table 1) which did not precipitate with anti kappa serum No inhibition was obtained with fraction 5 which precipitated with anti kappa but not with anti IgA

DISCUSSION

Receptors for human IgA on *group A streptococci* were shown in 1975 by *Christensen & Oxelius* (5) The IgA receptor of type 4 streptococci differed from the IgG receptor present on this streptococcal type as shown in experiments with uptake on whole bacteria of radiolabelled immunoglobulins (5) and with solubilized receptors (12) Group A streptococcal IgA receptors have affinity for both subclasses of human IgA (10)

The present results demonstrated that all of 8 tested freshly isolated type 4 group A streptococci bound between 60 and 80% of radiolabelled IgA1 M component in contrast to the reference strain type *M4 SS241* with an uptake of about 20% The SS241 strain had been subcultivated and kept for several years in the laboratory In inhibition experiments using polyclonal IgA and IgG as well as IgA and IgG M components only IgA was found to inhibit the binding which tallies with earlier results (5 10)

Gonococci were able to digest monoclonal IgA1 which confirms the findings by *Plaut et al* (11) The electrophoretic fractions containing IgA Fc fragments strongly inhibited the uptake of undigested radiolabelled IgA in the two type 4 strains tested while the fractions containing Fab but not Fc fragments were non inhibitory Similar results were obtained using fractions from gel filtration experiments of digested IgA myeloma protein Thus the data clearly demonstrated that IgA interacts with the streptococcal receptor via the Fc fragment

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IMMUNOGLOBULINS AND ALBUMIN IN SPUTUM FROM PATIENTS WITH CYSTIC FIBROSIS

A Study of Protein Stability and Presence of Proteases

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Schiøtz P O Clemmensen I & Høiby N Immunoglobulins and albumin in sputum from patients with cystic fibrosis A study of stability and presence of proteases Acta path microbiol scand Sect C 88 275–280 1980

Sputum sol phase from seventeen cystic fibrosis (CF) patients chronically infected in the lungs with mucoid *Pseudomonas aeruginosa* and presenting multiple precipitins in serum against this bacterium (CF + P) and 11 CF patients without *P aeruginosa* infection (CF P) were examined for proteolytic activity in a fibrin plate assay The proteolytic activity was significantly higher ($p < 0.02$) in sputum from CF + P patients than in sputum from CF patients This difference was only quantitative since sputum sol phase from both groups degraded fibrinogen to non precipitable material The proteolytic degradation of IgG IgA secretory IgA and albumin in the sputum sol phases was investigated by means of gel filtration and the stability of these proteins during various storage conditions was examined Degradation of IgG IgA secretory IgA and albumin in the sputa was not demonstrable and the proteins were stable for at least 4 weeks at 4 °C

Key words Cystic fibrosis sputum proteolysis antibodies

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Immunoelectrophoretic methods provide a variety of possibilities for quantitation or semiquantitation of different plasma proteins (24) By these methods it is also possible to quantitate proteins in inflammatory exudates (3) However these methods with the exception of crossed immunoelectrophoresis give no information as to whether the proteins are degraded or not This is important especially when studying inflammatory exudates with the presence of enzymes derived from both the inflammatory cells and bacteria

Crossed immunoelectrophoresis however is

fibrinogen) degradation products (4 6 16)

We have investigated the proteolytic activity in sputum from cystic fibrosis (CF) patients and its effect on fibrinogen and the immunoglobulins IgG and IgA responsible for the antibacterial antibodies measured in our previous studies (19 21 22) Albumin was included in the study as a sputum/serum albumin ratio has been used to measure transsudation of serum proteins to sputum (20)

MATERIAL AND METHODS

Patients

Twenty eight CF patients were included in the study (15 females and 13 males median age 14 years range 8–25) All patients had a typical history of CF and markedly elevated sweat electrolytes in repeated tests (7) The patients have been followed as previously described (9)

(i) One group consisted of 17 CF patients (8 males and 9 females median age 13 years range 8–25) suffering from chronic infection with mucoid strains of *Pseudomonas aeruginosa* (CF + P) and exhibiting more than 10 different precipitins in serum against water soluble antigens from these bacteria (10)

(ii) The other group consisted of 11 CF patients (five males six females median age 15 years range 11–22) without *Pseudomonas aeruginosa* lung infection (CF–P) and without serum precipitins against *Pseudomonas aeruginosa* antigens In seven of the CF P patients *Staphylococcus aureus Haemophilus influenzae* or *Escherichia coli* were isolated from the respiratory tract during this study

Sputum

A 3 h sputum sample was collected between 8 a m and 11 a m from each patient at 4 °C (to minimize proteolytic activity) as previously described (19) Each sample was subjected to bacteriological examination and the origin of the specimens from the lower respiratory tract was confirmed by studying the epithelial cells

Crossed Immunoelectrophoresis

This was performed as previously described (4)

Estimation of Proteolytic Activity in Sputum Sol Phase

This was done on both plasminogen rich and plasminogen poor fibrin plates as described by *Brakman & Astrup* (1)

Aliquots of 5 µl sputum sol phase or saline as control were incubated on the fibrin plate at 37 °C for 17 h The area of the lyzed zones is a measure of the proteolytic activity This was arbitrarily expressed as the product in square millimeters of two perpendicular diameters All samples were tested in duplicate and the average values were used as an arbitrary expression of proteolytic activity

Degradation of Fibrinogen by Sputum Sol Phase

200 µl human fibrinogen (4 4 µmol/l) in Tris HCl 0 05 M + 0 1 M NaCl pH 7 4 or 50 µl plasma (containing 1 6 µmol fibrinogen) was incubated with 200 µl sputum sol phase at 37 °C From the incubated material 10 µl samples were taken at different time intervals and tested in crossed immunoelectrophoresis against anti fibrinogen immunoglobulin (4)

Gel Filtration

Gel filtration was performed on Ultrogel AcA 34 columns (LKB Stockholm Sweden) equilibrated and subsequently eluted with phosphate buffered saline (PBS) 0 1 M at 4 °C A constant upward flow was used and eluted protein fractions were characterized by their partition coefficient $K_{AV} = (V_e-V_o)/(V_t-V_o)$ where V_e is the elution volume of the protein fraction V_o is the void volume and V_t is the total volume of the gel bed

Three different sputum sol phases with high proteolytic activity were examined Albumin IgG IgA and secretory IgA were investigated for degradation by determining the K_{AV} value for each eluted protein and comparing it with the value obtained by gel filtration of the corresponding sera

Quantitation of Protein

Albumin IgG IgA and secretory IgA concentrations were determined by rocket immunoelectrophoresis (26) The secretory IgA used as standard was isolated from human colostrum (23) The antisera were from Dakopatts Copenhagen except anti IgA which was from Behring Werke Marburg West Germany The analytical variation for protein determinations on sputum sol phase was calculated for each protein using the formula

$$SD = \sqrt{\frac{\Sigma d^2}{2n}}$$

where Σd^2 is the sum of the squared differences of double determinations of samples and n is the number of determinations (26) For each protein a total of 10 double determinations performed on different days and covering the actual concentration range were performed The analytical coefficients of variation were for albumin 6% IgG 6% IgA 10% and secretory IgA 8%

Estimation of Stability of Proteins during Storage

The protein stability in three sputum sol phases with

IgA at day zero and again after 1 2 4 and 8 weeks

Glycoprotein

The sol phase of mucus secretions is known to contain glycoproteins (5 18) For absorption of glycoprotein from sputum sol phase a Concanavalin A (Con A) (Pharmacia) solution (2 4 g %) was mixed with sol phase (1 1) incubated for 30 min at 20 °C and the samples were examined in rocket immunoelectrophoresis without antibodies in the agarose gel As one glycoprotein in the sol phase formed non immune precipitates in rocket immunoelectrophoresis the elution volume for the glycoprotein (see gel filtration) could be calculated and the K_{AV} value determined

RESULTS

Fibrinogen or Fibrinogen Degradation Products in Sputum Sol Phase

No fibrinogen antigenic material was found in sputum sol phase either by crossed immunoelectro

A

B

C

D

E

Fig 1 Pattern of immunoprecipitates obtained by crossed immunoelectrophoresis at pH 8 6 of human fibrinogen incubated with sputum sol phase at 37 °C for different periods of time The second dimension electrophoresis (anode at top) was run into agarose gel containing antibody to fibrinogen (A B C D & E) The material applied in the slit in the first dimension electrophoresis (anode to the right) was human fibrinogen incubated with buffer for 24 h (A) and with sputum sol phase for 5 min (B) 3 h (C) 6 h (D) and 24 h (E)

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phoresis or by quantitative rocket immunoelectrophoresis

Proteolytic Activity in Sputum Sol Phase as Estimated on Fibrin Plates

All sputum samples except one were found to degrade fibrin but the proteolytic activity differed in the two groups of patients In sputum sol phase from CF + P patients it was on an average 127 arb units/5μl (range 289–49) and this was significantly higher (p < 0 02) ...

results on plasminogen rich and plasminogen poor fibrin plates were identical

Incubation of Fibrinogen with Sputum Sol Phase

Not only fibrin but also fibrinogen was degraded by sputum sol phase (Fig 1) The degradation of fibrinogen was rapid as demonstrated by crossed immunoelectrophoresis (Fig 1B) Different fibrinogen degradation products were seen after various time of incubation (Fig 1C–D) and after 24 h incubation virtually all fibrinogen was degraded to non precipitable material (Fig 1E)

Incubation of normal plasma with a final concentration of fibrinogen as in Fig 1 with sputum sol phase caused no detectable fibrinogen degradation (Fig 2 A–D)

Gel Filtration of Sputum and Serum

The three sputum sol phases used for gel filtration had a proteolytic activity of 169 144 and 121 arb units respectively The K_{AV} values calculated for albumin IgA and IgG were identical for the three sputum sol phases and to the values obtained by gel filtration of serum (*Table 1*) The elution profile of the proteins was symmetrical indicating a homogenous protein Crossed immunoelectrophoresis of sputum sol phase also indicated that albumin IgG IgA and secretory IgA were not degraded



Fig 2 Pattern of immunoprecipitates obtained by crossed immunoelectrophoresis at pH 8 6 of normal human plasma incubated with sputum sol phase at 37 °C for different periods of time The second dimension electrophoresis (anode at top) was run into agarose gel containing antibody to fibrinogen (A, B and C) The material applied in the slit in the first dimension electrophoresis (anode to the right) was plasma incubated with sputum sol phase for 0 h 6 h and 24 h (A, B and C)

Stability of Albumin, IgG, IgA and Secretory IgA in Sputum

The stability of the four proteins in three sputum sol phases with high proteolytic activity i e 289, 225 and 196 arb units respectively, was examined by rocket immunoelectrophoresis The four proteins were all stable during storage at −20 °C as well as −80 °C for at least 2 months (Fig 3) IgG was stable for one month at 4 °C, the other proteins for at least two months, whereas all the proteins were unstable at room temperature

Non-Specific Precipitate in Agarose Caused by a Glycoprotein in Sputum

Sputum sol phase contains a glycoprotein, which migrates slightly anodically at pH 8 6 and forms a small precipitate inside the immune precipitate (Fig 4) The glycoprotein precipitate is formed also in agarose gels devoid of antibodies The precipitate is stained by the periodic-acid-Schiff reaction (polysaccharides) as well as by Coomassie Brilliant Blue (proteins) It is not removed in the routine washing procedure, used to remove non-precipitated proteins, but it can be removed by binding to Con-A (fig 4)

TABLE 1 *Average K_{AV} Values for Albumin IgG and IgA in Sputum Sol Phase and Serum from 3 Patients with Cystic Fibrosis Secretory IgA and Glycoprotein was only Analysed in the Sputa*

	K_{AV}-values	
	Serum	Sputum sol phase
Albumin	0 64	0 64
IgG	0 48	0 44
IgA	0 40	0 40
Secretory-IgA	–	0 24
Glycoprotein	–	0 68

DISCUSSION

Bronchial lavage as well as expectorated sputum has been used in immunochemical analyses of proteins from bronchial secretions (2, 12, 14, 17) However, to our knowledge, it has never been investigated whether the proteolytic activity, often present in bronchial secretions (13), has partially degraded the proteins and thus invalidated the results

The present study shows that chronic Pseudomo-

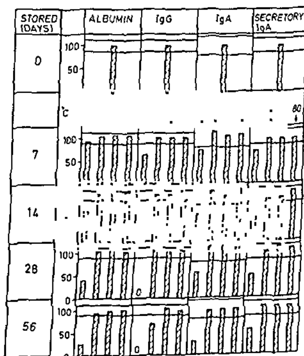


Fig 3 Albumin IgG IgA and secretory-IgA concentrations measured by rocket immunoelectrophoresis in sputum sol phase stored for 8 weeks at different temperatures The concentrations are average values from 3 sputum sol phase samples with high proteolytic activity They are expressed in % of the average concentration at day 0 The ± 2SD of analysis on double determinations are indicated with horizontal bars

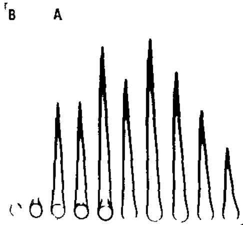


Fig 4 A Demonstration of glycoprotein precipitate in routine albumin determination in sputum sol phase by rocket immunoelectrophoresis Wells 1–8 from right 1–4) dilutions of standard serum 1 1200 1 600 1 400 1 300 5) control serum diluted 1 600 6–7) sputum sol phase d luted 1 9 and 1 19 8) sputum sol phase after absorption with Con A dilution 1 19

Antibody Rabbit antihuman albumin corresponding to 0 9 μl/cm² gel area

B Wells 1–2 from right same as A7 and 8 No antibody in the gel

nas aeruginosa lung infection in CF patients is associated with a high degree of proteolytic activity in sputum This is probably an expression of the severe inflammation which takes place in the lungs in CF + P patients and is in accordance with the poor prognosis for CF patients with chronic *Pseudomonas aeruginosa* lung infection (11)

The proteolytic enzymes in sputum not only degrade fibrin but also fibrinogen (Fig 1) The degradation of fibrin by sputum to non precipitable peptides is more extensive than could be obtained by the fibrinolytic enzyme plasmin (4) This is in agreement with results obtained by tryptic degradation of fibrinogen (15) Fibrin(ogen) degradation products are ofte found in inflammatory exudates and their presence can likely be ascribed to the activity of enzymes released from inflammatory cells (4) Whether the enzymes in sputum primarily originate from bacterial or inflammatory cells remains to be elucidated Also the content of protease inhibitors especially the ›low molecular weight bronchial protease inhibitor« described by *Hochstrasser et al* (8) remains to be determined in CF patients

The high proteolytic activity found in the sputum sol phase from CF patients and notably CF + P patients does however not degrade albumin IgG IgA or secretory IgA to a measurable degree in vivo as estimated from their K_{AV} values To avoid in vitro degradation of the proteins during collection the patients should expectorate in a thermobox placed at 4 °C as suggested by *Lopez Vidriero et al* (14) Sputum samples examined within four weeks can be stored at 4 °C without detectable degradation Only the stability of albumin IgG IgA and secretory IgA has been measured Examination of more labile proteins (like complement components) undoubtedly requires freezing during storage

The glycoprotein demonstrated in the sputum sol phase has a molecular weight around 60 000 calculated from its K_{AV} value The mol wt its staining properties and the absorption experiments with Con A indicates that it is a glycoprotein It probably reacts non specifically with agarose in a similar manner as does Con A with α D-glucopyranoside residues

Radial immunodiffusion and similar techniques have been used in the determination of protein concentrations in sputum sol phase (25) and this can lead to misinterpretations because the above mentioned glycoprotein forms a precipitate around the central well in the agarose gel This problem can be avoided by use of control plates without antibodies in the gel which will reveal the non specific nature of the precipitates and by electroimmunoassay as the morphology of the precipitate is different from immune specific precipitates (19 22)

In conclusion this study shows that sputum sol phase is suitable for examination of albumin IgG IgA and secretory IgA with immunoelectrophoretic methods if the samples are collected stored and analysed as described above

This work was supported by *The Danish Medical Research Council* (project nr 512 5267 6623 and 8115) *The National Association for the Fight against Tuberculosis The National Danish Association against Cystic Fibrosis* and *The National Danish Association*

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Acta path microbiol scand Sect C 88 281–286 1980

HEREDITARY NEUROLOGIC DISORDERS, CHARACTERIZED BY ATAXIA

Immunological in vitro *Parameters and HLA*

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Pedersen L Platz P & Raun N E Hereditary neurologic disorders characterized by ataxia Immunological *in vitro* parameters and HLA Acta path microbiol scand Sect C 88 281–286 1980

Nineteen unrelated patients with hereditary ataxia hereditary spastic paraplegia and Charcot Marie Tooth s syndrome were investigated for immunological *in vitro* parameters (lymphocyte transformation with PHA con A PWM antigens determination of T and B lymphocytes and HLA typing) When compared with normal controls no significant deviation was found

Key words Hereditary neurologic disorders ataxia HLA immunological parameters

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Hereditary ataxia (HA) is a group of progressive and disabling disorders due to degeneration in cerebellum brainstem posterior columns of the spinal cord and the spinocerebellar tracts They have clinical resemblance to and can present differential diagnostic problems to other degenerative or demyelinating disorders such as Creutzfelt Jakobs (CJD) ataxia teleangiectasia (AT) and multiple sclerosis (MS) Although all the latter diseases may show familial clustering they rarely present the patterns of inheritance which are characteristic for HA

Genetic factors within the HLA system especially Dw2 have firmly been demonstrated to increase the susceptibility to MS (12) and recently *Jackson et al* (11) found in one large family that HA was genetically linked to the HLA system Linkage to HLA could however not be confirmed by *Wastiaux et al* (20) We have therefore examined several families with different forms of HA in order to investigate whether immunological parameters or the HLA system could help to elucidate the pathogenesis or provide genetic markers in these disorders In this paper we report the immunological results and HLA types on unrelated propositi detailed pedigrees and linkage studies will be reported elsewhere

MATERIALS AND METHODS

Patients Material

The patients are the propositi from 19 families two of these families were later traced to be connected 4 generations back The propositi were collected among hospital admissions for hereditary ataxia hereditary spastic paraplegia (HSP) and Charcot Marie Tooth (CMT) in the eastern part of Denmark (2 mill inhabitants) and in the period 1961–1975 They represent 1/3 of families with these diagnoses (HA) in this area Results of the epidemiological survey will be published elsewhere All patients in the material were examined and the clinical diagnosis established before the investigation Extensive family histories including

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TABLE 2 *HLA ABC phenotypes and Genotypes for 19 Patients with Hereditary Ataxia*

Diagnosis	Phenotype	Genotype
CA	A 1 2 B 7 8	
	A 1 – B 8 14	HLA A 1 B8/A 1 B 14
	A 1 2 B 5 17	HLA A 1 B 17/A 2 B 5
	A 1 11 B 5 7	HLA A 11 B 5/A 1 B 7
	A 2 – B 12 27 C 2	HLA A 2 B 12/A 2 B 27 C 2
	A 1 9 B 8 44 C 4	
FA	A 3 – B 7 27 C 2	
	A 1 3 B 8 35 C 4	
	A 2 3 B 5 7 C 2	HLA A 2 B 5 C 2/A 3 B 7
	A 1 2 B 8 12	HLA A 2 B 12/A 1 B 8
HSP	A 2 11 B 7 40 C 2	
	A 1 – B 7 8	HLA A 1 B 8/A 1 B 7
	A 2 26 B 12 –	HLA A 26 B 12/A 2 B 12
	A 1 2 B 8 50	HLA A 1 B 8/A 2 B 50
	A 2 3 B 15 35 C 3 4	
CMT	A 1 2 B 8 40 C 2	HLA A 1 B 8/A 2 B 40 C 2
	A 24 32 B 7 27 C 2	HLA A 24 B 7/A 32 B 27 C 2
	A 2 9 B 7 14	
	A 3 9 B 16 39	

For 8 patients genotypes are not given due to lack of family at time of investigation

variance while homogeneity of variances was tested by Bartlett Box F value For comparisons between HA patients and controls t test was used

RESULTS

HLA ABC phenotypes and genotypes for each patient are seen in Table 2

HLA typing of the 19 proposities revealed no striking deviation (Table 3) and after correction for the ...

Lymphocyte transformation tests (Table 4) did not show any significant deviation from the controls either with mitogens or with antigens PHA and con A was used in 3 different concentrations Only the optimum value is given in Table 4 but the other concentrations gave also normal values

Determination of lymphocyte subpopulations (Table 5) showed a slight but significant decrease of EAC rosetting lymphocytes when compared to the standard control panel which was collected during a one year period When however only controls investigated in the same 2 months July and August as the patient groups were considered no significant differences were seen

DISCUSSION

The clinical distinction between MS and single cases of HA has always been difficult (1 8 10) and among our 19 patients 4 were previously classified as having MS Their diagnosis was changed when it was realized that they were members of confirmed HA families

That 20 per cent of our patients although belonging to families with HA had been diagnosed as MS-cases and even 3 of them as familial MS cases stresses the importance of extensive family histories in any familial neurologic disorder The immunological *in vitro* tests showed that the general immune competence in HA patients is norm...

... cytes when compared with the whole control group but only a slight difference

searches in church and birth registries were made on all families

Many authors have described subgroups with various eponyms within HA. A few are based only on clinical or patho-anatomical descriptions of a single family. The distinction between the following diagnoses: Hereditary spastic ataxia (Sanger Brown), spino-pontin atrophy (Boller Segarra), olivo-cerebellar atrophy (Holmes) and olivo-ponto-cerebellar atrophy (Menzel) is difficult on clinical grounds. The onset occurs in the same decades and the transmission is nearly always dominant (5, 6, 7). For use in this study we have therefore grouped the small entities as one, called cerebellar ataxia (CA). However, cases of Friedreich ataxia (FA) were considered a separate entity, characterized by onset of disease before the third decade, recessive transmission and symptoms from the brainstem and posterior columns.

HSP and CMT are rather distinct disorders closely connected to HA.

Thus we have 4 reasonably distinguishable groups of patients: CA, FA, HSP and CMT, for which the clinical data appear in Table 1.

Immunological Methods

HLA typing for the HLA-ABC antigens was performed by the micro-lymphocytotoxic method as described by Kissmeyer-Nielsen & Kjerbye (13).

Estimation of lymphocyte subpopulation and lymphocyte transformation has previously been described in detail (17). In brief: Peripheral blood was drawn in equal volume of medium RPMI 1640 with 15 i.e. Heparin/ml. For E and EAC rosetting, the blood medium mixture was incubated with carbonyl iron for 30 min at 37 °C; iron and phagocyting cells were removed with a strong magnet. For E rosetting, lymphocytes were incubated with sheep red blood cells (SRBC) for 30 min at 37 °C followed by overnight incubation at 4 °C. EAC rosetting lymphocytes were determined with human blood group A erythrocytes coated with rabbit anti-A and mouse complement. Both E and EAC rosettes were gently resuspended and counted in a haemocytometer; rosettes were defined as lymphocytes with 3 or more red cells attached. All tests were performed in duplicate.

Surface membrane immunoglobulin (SmIg) was determined with a FITC conjugated rabbit anti-human immunoglobulin (Dakopats code no. 1009). The fluorescent lymphocytes were read and counted in a Leitz Orthoplan microscope. Phagocyting cells were identified by Latex incubation but not counted.

Lymphocyte transformation was performed as previously described (17). Mononuclear cells were isolated on Lymphoprep, washed in medium and set up in triplicate cultures in Nunc tubes. Each tube contained 10^5 cells in 0.5 ml of culture medium consisting of RPMI 1640 supplement with 25 mM Hepes, glutamine, antibiotics and 15 per cent pooled serum from random non-transfused male blood donors. The cells were cultured at 37 °C in a humidified atmosphere containing 5 per cent CO_2 and the cultures were harvested after 96 h (mitogen stimulated cultures) or 120 h (antigen stimulated cultures). Twenty-four hours before termination of culture 0.05 μCi ^{14}C-thymidine was added to each culture. The cells were harvested by a Skatron semi-automatic harvester on glass fibre filters and washed with distilled water. The filters were counted in vials containing 2.5 ml of Instagel in a Beckman liquid scintillation counter. The mitogens employed were: Phytohaemagglutinin (PHA-P, Difco) – final dilution 1:600, concavallin (con A) – 1:20, pokeweed mitogen (PWM, Gibco) – 1:500. The antigens were: Purified protein derivative (PPD) without chinosol (State Serum Institute, Copenhagen) – protein concentration 1 μg/ml; *Candida albicans* protein concentration 2 mg/ml; *Escherichia coli* and *Staphylococcus aureus* – (heat killed organisms prepared by Dr. Klaus Jensen) – 10^6 organisms/ml.

Statistical Methods

To obtain gaussian distribution, square root transformation was applied to lymphocyte transformation countings. Mean values for the 4 disease groups (CA, FA, HSP, CMT) were compared by one way analysis of

TABLE 1. *Clinical Data on 19 Unrelated Patients with Hereditary Ataxia*

N	Median duration of disease in years (range)	Median age of onset (range)
CA 6	16 (11–23)	34 (24–45)
FA 4	21 (16–45)	16 (14–23)
HSP 5	26 (21–39)	27 (22–36)
CMT 4	39 (2–41)	7 (2–38)

CA Cerebellar ataxia
FA Friedreich's ataxia
HSP Hereditary spastic paraplegia
CMT Charcot-Marie-Tooth's disease

TABLE 5 *Lymphocyte Subpopulations in Patients with HA and Normal Individuals*

	N	Per cent E rosettes	Per cent EAC rosettes	Per cent SmIg	N	Total lymphocytes x10⁹/l
HA	19	57 2 ± 1 7	8 7ᵃ ± 0 6	15 3 ± 1 1	11	2 21 ± 0 22
Controls I	64	60 7 ± 1 1	13 3 ± 0 5	14 3 ± 0 4	49	2 39 ± 0 12
Controls II	22	56 8 ± 2 01	10 9 ± 0 7	14 0 ± 0 91		

Mean values ± one standard error of mean
Control group I Reference group investigated prior to investigation of HA patients
Control group II Normal individuals investigated during the same 2 months as the HA patients
ᵃ < 01 when compared to control I
n s when compared to control II
N number of individuals investigated

must therefore await combination with other series

We have not been able to confirm the report of Nese *et al* (16) who suggested an association between HLA Bw35 Cw4 Dw1 and FA Only one of 4 patients with FA was HLA Bw35 positive

As seen in Table I our material comprised small groups but as no deviation was found between the single groups of CA FA HSP and CMT we considered them as one group These groups are not clinically clearly distinguishable Many reported families show great variation both in phenotype and patho-anatomically including both cases of typical HA and cases resembling HSP or CMT (3 14 18)

Mavranthopoulos & Smith (15) claimed a connection between many hereditary degenerative disorders forming a continuum including Mb Alzheimer Creutzfelt Jakobs disease (CJD) and amyotrophic lateral sclerosis (ALS) in the familial form It is interesting that CJD now known as a transmissible disease (2 4) has appeared in multiple generations with an autosomal dominant inheritance pattern leading to suspicion of a genetic deficiency determing the susceptibility to a latent virus (9)

In this neurologic continuum a certain coherence between genetics immunology and disease susceptibility seems probable but we have not been able to demonstrate any immunodeficiency in this small number of patients with HA

Still a genetic susceptibility might be related to the histocompatibility complex and the possible genetic linkage between the HLA-complex and HA is being investigated further Preliminary results have not given evidence of any strong linkage

This study was aided by grants from *Warwara Larsen s Foundation the Danish Multiple Sclerosis Society* and the *Medical Research Council*

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TABLE 3 *HLA Antigen Frequencies in Unrelated Patients with Hereditary Ataxia and in the Danish Population*

ANTIGENS	Hereditary ataxia N = 19 per cent positive	Controls N = 1967 per cent positive
HLA-A1	52 6	31 1
HLA-A2	57 9	53 6
HLA-A3	26 3	26 9
HLA-A9	21 1	17 3
HLA-A10	5 3	9 6
HLA-A11	10 5	10 1
Hla A19	5 3	18 1
HLA-B5	15 8	10 6
HLA-B7	42 1	26 8
HLA-B8	42 1	23 7
HLA-B12	26 3	25 2
HLA B13	0	4 3
HLA-B14	10 5	4 5
HLA-B18	0	7 1
HLA-B27	15 8	8 6
HLA-Bw15	5 3	17 9
HLA-Bw16	5 3	5 4
HLA Bw17	5 3	7 7
HLA Bw21	5 3	3 5
HLA Bw22	0	3 8
HLA-Bw35	10 5	13 1
HLA Bw37	0	2 1
HLA Bw40	10 5	17 9
HLA Cw1	0	5 7
HLA Cw2	31 6	9 7
HLA Cw3	5 3	35 0
HLA Cw4	15 8	17 1

when compared to controls investigated in the same period This difference may be due to technical variation in performance of the test but perhaps more likely due to seasonal variation in EAC rosetting lymphocytes in normals and illustrates the importance of simultaneous controls

In this study although the numbers are small no significant deviations of HLA frequencies were seen HLA typing of unrelated patients with HA has to our knowledge not previously been reported nor registered in the HLA and Disease Registry (19)

A definite answer of HLA association with HA

TABLE 4 *Lymphocyte Transformation with Mitogens and Antigens*

	HA N = 19	Controls N = 150
PHA 40 μg ml	32833 ± 2060	35588 ± 1299
PWM	8992 ± 845	8592 ± 390
con A 1 20	15575 ± 700	18728 ± 735
PPD 10 μg ml	7044 ± 1069	6745 ± 455
Cand albicans	2505 ± 388	2675 ± 175
Staph aureus	1687 ± 299	2300 ± 195
E coli	1316 ± 288	1422 ± 131

Mean values in cmp ± one standard error of mean
Only results obtained with optimal concentrations of PHA and con A are given

ENCEPHALITIS INDUCED IN RABBITS BY STAPHYLOCOCCAL LIPOTEICHOIC ACID

PER AASJORD HARALD NYLAND and SVERRE MØRK

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Aasjord P Nyland H & Mørk S Encephalitis induced in rabbits by staphylococcal lipoteichoic acid Acta path microbiol scand Sect C 88 287–291 1980

Rabbits were immunized with staphylococcal lipoteichoic acid (LTA) in Freund s adjuvant After four injections (six weeks) the rabbits showed decreased activity and unsteadiness of the head Two weeks after the sixth injection (ten weeks) two of five rabbits developed clinical signs of encephalitis with nystagmus ataxia general weakness decreased activity and dragging of the hind legs The other three animals showed only mild symptoms Neuropathological examination showed inflammatory infiltrates containing small lymphocytes and some plasma cells in the leptomeninges and within the perivascular spaces of the brain

Key words Lipoteichoic acid encephalitis

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Experimental allergic encephalomyelitis (EAE) is an inflammatory condition of the central nervous system (CNS) which can be induced in animals by sensitization with CNS tissue (1) The encephalitogenic property resides in the myelin basic protein (MBP) and several encephalitogenic determinants have been defined (17) Differences in antigenic composition type of adjuvant mode of antigen administration and animal species and strains account for the differences in the clinical manifestations of EAE varying from hyperacute to chronic disease (1 15) The histopathological lesions consist of perivascular inflammatory infiltrates and demyelination (14) EAE has been used as a model system for an autoimmune type of demyelination (1)

The immunogenic properties of staphylococcal lipoteichoic acid (LTA) were described in an earlier paper (21) Th[illegible]

[illegible] Cowan I The possible significance of this observation in relation to multiple sclerosis (MS) is discussed

MATERIALS AND METHODS

Strain and Growth

[illegible] previously described (20)

Antigen Preparations

LTA was prepared according to the method of *Coley et al* (3) with minor modifications Bacteria were suspended in water (2 ml per g of bacteria) and mixed with an equal volume of 90 per cent phenol The suspension was incubated during vibration (Vibrio Mischer type 1 Bopp & Reuter BRD) for 30 min at 65 °C cooled on ice and centrifuged (5 min at 20 000 × g) The [illegible]

[illegible] ammonium acetate pH 6 9 containing 0 02 per cent azide as eluate The composition of LTA was as estimated in (20)

Protein A and peptidoglycan were prepared as described in (6) and (9) respectively

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Administration of Antigen

Five New Zealand White rabbits of the institute's own breed were used as test animals. A mixture of LTA in 0.5 ml isotonic saline and 0.5 ml Freund's complete adjuvant (Difco Laboratories, Detroit, Mich., USA) was injected intramuscularly into the hind leg. The following injections were a mixture of LTA and Freund's incomplete adjuvant (Difco). Each dose contained 2 mg of antigen and the injections were given at intervals of two weeks. Each rabbit received a total of 12 mg.

Two non-immunized rabbits matched in age with the experimental animals, one rabbit immunized with protein A and one with peptidoglycan in adjuvant, served as controls.

Neurological Observations

The animals were studied daily for possible neurological signs. Two weeks after the sixth injection, the rabbits which showed symptoms of encephalitis were killed.

Neuropathological Examination

The brain and spinal cord were removed and fixed in 4 per cent formaldehyde for seven days. For light microscopy, coronal sections (12 μm) of the brain and spinal cord from paraffin embedded blocks were prepared on a Model Hn 40 Microtome (Young AG, Heidelberg, BRD) and stained with hematoxylin (E. Merck AG, Darmstadt, BRD) and eosin (Difco, West Molefey, UK), followed by cresyl violet (Merck) and luxol fast blue (Pfaltz & Bauer Inc., Flushing, N.Y., USA). The control rabbits were dissected and examined in the same way as the LTA injected animals.

Brain tissue from one autopsy case of acute MS was prepared as above and examined histologically.

Antisera and Serological Tests

Antisera to LTA were produced in rabbits as described for administration of antigen. Anti-protein A and anti-peptidoglycan were prepared as described in (7) and (10), respectively.

All sera and antigens were examined against each other by the double diffusion in agar test (8).

RESULTS

The double diffusion in agar test showed that the LTA preparation used in the experiments did not produce precipitation lines with protein A or with peptidoglycan from *S. aureus*. The anti-LTA antibody titres in the rabbit sera varied from 640 to 2560 (21).

Two days after the fourth injection (six weeks) two of the rabbits showed minor clinical symptoms with decreased motor activity and unsteadiness of the head with slow turning movements. The symptoms gradually disappeared after one week but returned two days after the fifth injection (eight weeks). Additional symptoms then appeared after the sixth injection (ten weeks) including [illegible]us, ataxia, general weakness and dragging of the hind legs. The other three rabbits showed only mild symptoms after the sixth injection with decreased activity and general weakness, but without any definite neurological signs.

The brain and spinal cord were macroscopically normal. Coronal sections of the brain showed patchy mononuclear inflammatory reactions in the leptomeninges (Fig. 1a) with extension along the Virchow-Robin space to the perivascular spaces of the neural parenchyma (Fig. 1b), mainly in the cortex. Small lymphocytes and plasma cells were the predominant cell types in the infiltrates. No spinal cord lesions were observed and no direct evidence for demyelination could be obtained. In the acute MS brain, the inflammatory lesions showed similarities with those found in the rabbit brains (Fig. 1c) but demyelination was prominent. Neither the untreated rabbits, nor the rabbits which had received other antigens in Freund's adjuvant showed any inflammatory reactions.

DISCUSSION

This report gives clinical and neuropathological evidence for the development of a meningoencephalitis in rabbits immunized with staphylococcal LTA. In two of the five animals, the clinical symptoms were similar to those of EAE (1). The other three showed only minor clinical signs. EAE commonly develops within one to four weeks after a single injection of MBP in complete Freund's adjuvant (1, 14). Our animals showed a different course with a) delayed appearance of symptoms, b) a gradual worsening related to repeated injections of the antigen, c) periodical fluctuation of the symptoms with exacerbation of symptoms two to four days after every renewed administration of antigen following the fourth injection. The neuropathological changes included inflammatory reactions in the leptomeninges and the perivascular spaces around the vessels in the neural parenchyma, which is consistent with EAE (14). No evidence was, however, obtained for presence of sleeve-like demyelination which is found in EAE.

It has been reported that inflammatory lesions w th dam[illegible]

[illegible] bacteria into the animal's brain. This effect appears to be a non-specific consequence of a delayed hypersensitivity reaction to mycobacteria. A cross reactivity between MBP and mycobacteria antigens has also been described, but injection of mycobacteria individually



Fig 1 Light microscopy of coronal sections of rabbit and human brains a) Mononuclear infiltrates in the leptomeninges from a LTA injected rabbit 250 x b) Extension of mononuclear infiltrates along a pia vessel extending from the leptomeninges into the brain from the same animal 250 x c) A small venule in the white matter from a human MS brain surrounded by mononuclear infiltrates 250 x

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do not produce signs of EAE (16) Recently EAE like conditions have been induced in animals by immunization with extracts from human bronchial carcinoma (5) and glioma (18) which did not contain MBP

In our laboratory neurological signs have never been observed in rabbits after immunization with several other staphylococcal antigens in Freund s adjuvant (complete and incomplete) for example protein A peptidoglycan and other less well defined substances LTA from other Gram positive bacteria may also induce encephalitis LTA from *Streptococcus mutans* has been shown to produce an intense inflammatory reaction when injected into the periodontal tissue of rats (2) *Ne eman & Ginsburg* (11) have induced arthritis in rabbits after immunization with streptococcal LTA and subsequent administration of the antigen into the joint Recently a nephritis were induced in animals hyperimmunized with streptococcal LTA coupled to albumin (4) Deposits of immune complexes of LTA and immunoglobulin were detected in the renal glomeruli *Ofek et al* (13) have shown that streptococcal LTA can react with MBP but they failed to induce encephalitis in rabbits when complexes of LTA and MBP were used for immunization This could be due to the short period of immunization (four weeks) or to the different bacterial species used for LTA extraction The reaction may also be confined to our strain of New Zealand White rabbits This has to be further investigated with other animals such as guinea pigs and rats and with other strains of rabbits

The gradual increasing and fluctuating neurological symptoms in the rabbits show similarities to those found in patients with MS The inflammatory reactions in the rabbit brains had similarities with the acute MS lesions in an autopsy case with a clinical history of one year The characteristic demyelination of the MS lesions was absent from the rabbit brain

Preliminary experiments on absorption of human cerebrospinal fluid with our LTA preparation showed that some of the electrophoretically most cathodic oligoclonal IgG bands disappeared from MS samples but not from control samples (12) Thus LTA may be implicated in the pathogenesis of multiple sclerosis

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LYMPHOCYTE SUBPOPULATIONS IN MULTIPLE SCLEROSIS VARIATIONS IN ROSETTE TESTS USING ERYTHROCYTES FROM DIFFERENT SHEEP

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Næss A & Nyland H Lymphocyte subpopulations in multiple sclerosis Variations in rosette tests using erythrocytes from different sheep Acta path microbiol scand Sect C 88 293–297 1980

Lymphocytes from 34 patients with active and with stable multiple sclerosis (MS) were examined by rosetting techniques Patients with active disease had a relative lymphopenia (35 ± 9 %) When tested with erythrocytes (E) from one out of three sheep used patients with active MS had a decrease in E rosette forming cells (E–RFC) compared to patients with stable disease and with healthy controls Other tests (active E– E_{AET}– EA– and EAC–RFC) did not disclose any differences between patients and controls The results suggest that the origin of the E may be of importance in E–RFC tests

Key Words Sheep erythrocytes rosette tests lymphocyte subpopulations multiple sclerosis

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Membrane marker techniques are a relatively simple method for the demonstration of lymphocyte subpopulations T lymphocytes have receptors for sheep erythrocytes (E) and form rosettes with these (E rosette forming cells E–RFC) A subpopulation *of E–RFC also form rosettes under suboptimal* conditions (low E lymphocyte ratio minimal incubation) These are usually termed »active« E–RFC *Their significance is unknown but they are* supposed to have more »avid« receptors for E than other E–RFC (34 37) Non–E–RFC include lymphocytes that will form rosettes with antibody (A) coated E (EA–RFC) by means of receptors for the Fc fragment of the immunoglobulin (7) and cells with receptors for the third component of complement (C) forming rosettes with erytrocyte antibody-complement complexes (EAC–RFC) (16) A small percentage of lymphocytes form rosettes with *more than one of these markers* No single study of lymphocyte subpopulations in multiple

decrease in E–RFC in peripheral blood from patients with active MS but not ... 23) The peripher ... of active ... have also compared a technique using 2 aminoethy lisothiouronium bromide hydrobromide (AET)-treated E (13) with the original technique using untreated E This test has been proposed as more reliable for T lymphocytes than the E–RFC test because of greater stability of the rosettes Since the results observed with the E–RFC test were different from those previously observed in this laboratory we used E from three different sheep for rosette formation in an *additional* experiment

TABLE 2 *Active E and Total E–RFC Tests Performed with E from 3 Different Sheep*

		Active E–RFC %				Total E–RFC %		
Active MS		A	B	C		A	B	C
Patient no	1	61	58	68		81	70	49
	2	56	53	57		73	60	57
	3	55	60	56		n d	n d	n d
	4	23	64	61		64	73	57
	5	40	44	33		62	71	62
	6	60	61	59		69	76	65
	7	70	62	69		65	80	62
	8	n d	n d	n d		82	64	58
	9	n d	n d	n d		70	61	44
	(n – 7)	52 ± 16 (23–70)	57 ± 7[a] (44–64)	58 ± 12 (33–69)	(n = 8)	71 ± 8[b] (62–82)	69 ± 7[c] (60–80)	57 ± 7[b c d e] (44–65)
Stable MS	(n = 6)	44 ± 13 (26–67)	42 ± 9[a] (26–50)	47 ± 13 (29–66)	(n = 8)	64 ± 11 (48–82)	62 ± 7 (51–72)	67 ± 6[e] (54–75)
Controls	(n – 11)	46 ± 16 (18–73)	52 ± 15 (29–73)	50 ± 12 (34–66)	(n 16)	68 ± 7[f] (58 82)	66 ± 9 (46–80)	64 ± 6[d f] (55–76)

Values are mean ± S D (range)
[a]) p 0 008 (active MS v stable MS)
[b]) p 0 03 (sheep A v sheep C)
[c]) p 0 002 (sheep B v sheep C)
[d]) p 0 02 (active MS v controls)
[e]) p 0 02 (active MS v stable MS)
[f]) p 0 04 (sheep A v sheep C)

– 0 08) for this group As this was somewhat in contrast to our previous results we decided to extend the investigation to include E–RFC tests with E from 3 different sheep When the active E–RFC and the total E–RFC tests were performed with E from 3 sheep a number of significant differences were observed (Table 2) In the active E–RFC test patients with active MS had significantly higher levels of RFC than patients with stable disease only when E from sheep B were used In the E–RFC test patients with active disease had a decreased percentage of RFC compared to patients with stable MS and controls only when tested with E from sheep C Accordingly E–RFC levels in patients with active MS were significantly lower when blood from sheep C was used rather than blood from A or B Seven out of 8 patients had lower E–RFC levels when tested with sheep C than with the other two sheep one had lower E–RFC counts with sheep A and C than with sheep B

DISCUSSION

The present study has demonstrated a decrease in the percentage of lymphocytes in peripheral blood from patients with active MS The same patient group showed a significant decrease in E–RFC percentage with E from one of the three sheep tested (C) In addition with E from sheep B there was a significant increase in active E–RFC percentage in [illegible]

[illegible] active MS this does not apply to the lymphocyte [illegible]

[illegible] patient groups are not similar however and the control values are also different from this study Also *Sagar & Allonby* (31) found significantly lower total lymphocyte counts in MS patients without any correlation to disease activity The E–RFC test has been used extensively in patients with MS and with conflicting *results* Some investigators who like ourselves have explicitly separated patients with active and stable MS have found decreased E–RFC percentages in active disease only (1 17 33) and *Huddlestone & Oldstone* (9) who followed 9 patients during exacerbation and remissions found lower E–RFC percentages during

MATERIALS AND METHODS

Patients Thirty four patients with MS were examined at the Department of Neurology The patients were assigned to one of two groups 1) those with *active* disease (n = 16) either a first attack or a recent exacerbation requiring hospital admission and 2) those with *stable* disease (n = 18) with no exacerbation during the 6 months preceeding admission

Controls Thirty two healthy persons with leukocyte counts between 4000 and 10 000/mm³ 20–50% lymphocytes were selected from among the hospital staff

Sheep erythrocytes were obtained from 3 different sheep (A B and C) The sheep were bled at the same time and the E preserved in Alsever s solution and washed twice in Hanks balanced salt solution (HBSS) before use

Lymphocyte studies Differential counts were per formed on May Grunwald Giemsa preparations from unseparated whole blood Lymphocyte concentrations were derived from total leukocyte counts and differential counts Lymphocyte separation was performed as described previously (23) Briefly defibrinated blood was mixed with an equal volume of phosphate buffered saline (PBS) and carefully layered upon Lymphoprep® After centrifugation at 400 *g* at the interface between blood and Lymphoprep® the interface layer containing the lymphocytes was pipetted off The cells were washed 3 times in HBSS and the concentration adjusted to 4×10^6/ml

Active E rosette forming cells (active E–RFC) were enumerated by adding 0 2 ml of 1% E to 0 2 ml of lymphocyte suspension centrifuging at 200 *g* for 5 min resuspending and counting (34) *E rosette forming cells (E–RFC)* were determined as described previously (23) A lymphocyte suspension of 0 2 ml was mixed with an equal volume of 0 5% E incubated for 5 min at 37 °C centrifuged for 5 min at 200 *g* and stored in ice water overnight before counting *E_{AET} rosette forming cells (E_{AET} RFC)* were prepared in the same way as E–RFC using 2 aminoethylisothiouronium bromide (AET)-treated E and 25% fetal calf serum (13) *EA rosette forming cells (EA–RFC)* were enumerated by an assay with ox erythrocytes (E) sensitized with rabbit IgG antibodies (A) (7) By this technique the presence on the cell of receptors for the Fc portion of IgG is demonstrated *EAC rosette forming cells (EAC–RFC)* were determined by incubating 0 2 ml of lymphocyte suspension with 0 2 ml of indicator cells (20) at 37 °C for 20 min resuspending and counting Cells having receptors for the C3b fraction of complement form rosettes by this method For all rosette preparations a minimum of 200 lymphocytes were counted at a magnification of 600× All samples were counted by the same person Lymphocytes binding more than *3 E* were counted as *RFC*

Statistics The statistical significance of the observed differences was established by Student s t test $p < 0.05$ was considered significant

RESULTS

The results are summarized in Table 1 and 2 There was no significant difference in leukocyte counts between the groups (Table 1) although both patients with active and with stable disease had slightly higher leukocyte counts than the controls In contrast the percentage of lymphocytes was significantly reduced in patients with active MS ($p = 0.002$) and slightly ($p = 0.09$) reduced in patients with stable disease As a consequence lymphocyte concentrations per mm³ were rather similar for the three groups The initial experiments did not disclose any significant differences between patients and controls with any of the rosette techniques used although patients with active disease had active E–RFC levels slightly increased ($p = 0.07$) compared both to patients with stable MS and to controls In contrast total E–RFC levels were slightly lower (p

TABLE 1 *Peripheral Blood Lymphocyte Subpopulations in MS*

Group	No	Leukocytes per mm³	Lymphocytes %	Lymphocytes per mm³	Rosette forming cells % active E	E	E_{AET}	EA	EAC
Active MS	16	6100 ± 1400 (4000–8600)	35 ± 9[a] (21–52)	2100 ± 500 (1500–3200)	57 ± 15 (27–81)	62 ± 10 (39–78)	75 ± 12 (41–87)	21 ± 10 (7–39)	30 ± 15 (8–58)
Stable MS	18	5900 ± 1700 (3000–9100)	38 ± 10 (19–63)	2300 ± 800 (900–3900)	47 ± 14 (21–72)	66 ± 7 (54–78)	74 ± 9 (57–89)	23 ± 8 (10–35)	32 ± 11 (13–51)
Controls	32	5400 ± 1500 (3200–10 000)	43 ± 9 (21–61)	2300 ± 800 (1200–4900)	48 ± 13 (26–72)	67 ± 9 (46–84)	74 ± 11 (44–92)	25 ± 10 (10–47)	30 ± 11 (10–49)

Values are mean ± S D (range)
a) p = 0 02 (v controls)

TABLE 2 *Active E and Total E–RFC Tests Performed with E from 3 Different Sheep*

		Active E–RFC %				Total E–RFC %		
Active MS		A	B	C		A	B	C
Patient no	1	61	58	68		81	70	49
	2	56	53	57		73	60	57
	3	55	60	56		n d	n d	n d
	4	23	64	61		64	73	57
	5	40	44	33		62	71	62
	6	60	61	59		69	76	65
	7	70	62	69		65	80	62
	8	n d	n d	n d		82	64	58
	9	n d	n d	n d		70	61	44
	(n – 7)	52 ± 16 (23–70)	57 ± 7^{a} (44–64)	58 ± 12 (33–69)	(n = 8)	71 ± 8^{b} (62–82)	69 ± 7^{c} (60–80)	$57 \pm 7^{b,c,d,e}$ (44–65)
Stable MS	(n – 6)	44 ± 13 (26–67)	42 ± 9^{a} (26–50)	47 ± 13 (29–66)	(n – 8)	64 ± 11 (48–82)	62 ± 7 (51–72)	67 ± 6^{e} (54–75)
Controls	(n – 11)	46 ± 16 (18 73)	52 ± 15 (29–73)	50 ± 12 (34–66)	(n 16)	68 ± 7^{f} (58 82)	66 ± 9 (46–80)	$64 \pm 6^{d,f}$ (55 76)

Values are mean ± S D (range)
a) p 0 008 (active MS v stable MS)
b) p 0 03 (sheep A v sheep C)
c) p 0 002 (sheep B v sheep C)
d) p 0 02 (active MS v controls)
e) p 0 02 (active MS v stable MS)
f) p 0 04 (sheep A v sheep C)

0 08) for this group As this was somewhat in contrast to our previous results we decided to extend the investigation to include E–RFC tests with E from 3 different sheep When the active E–RFC and the total E–RFC tests were performed with E from 3 sheep a number of significant differences were observed (Table 2) In the active E–RFC test patients with active MS had significantly higher levels of RFC than patients with stable disease only when E from sheep B were used In the E–RFC test patients with active disease had a decreased percentage of RFC compared to patients with stable MS and controls only when tested with E from sheep C Accordingly E–RFC levels in patients with active MS were significantly lower when blood from sheep C was used rather than blood from A or B Seven out of 8 patients had lower E–RFC levels when tested with sheep C than with the other two sheep one had lower E–RFC counts with sheep A and C than with sheep B

DISCUSSION

The present study has demonstrated a decrease in the percentage of lymphocytes in peripheral blood from patients with active MS The same patient group showed a significant decrease in E–RFC percentage with E from one of the three sheep tested (C) In addition with E from sheep B there was a significant increase in active E–RFC percentage in lymphocytes from patients with active as compared to those with stable MS

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MATERIALS AND METHODS

Patients Thirty four patients with MS were examined at the Department of Neurology The patients were assigned to one of two groups 1) those with *active disease (n = 16)* either a first attack or a recent exacerbation requiring hospital admission and 2) those with *stable* disease (n = 18) with no exacerbation during the 6 months preceeding admission

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TABLE 1 *Peripheral Blood Lymphocyte Subpopulations in MS*

Group	No	Leukocytes per mm³	Lymphocytes %	Lymphocytes per mm³	Rosette forming cells % active E	E	E_{AET}	EA	EAC
Active MS	16	6100 ± 1400 (4000–8600)	35 ± 9[a] (21–52)	2100 ± 500 (1500–3200)	57 ± 15 (27–81)	62 ± 10 (39–78)	75 ± 12 (41–87)	21 ± 10 (7–39)	30 ± 15 (8–58)
Stable MS	18	5900 ± 1700 (3000 9100)	38 ± 10 (19–63)	2300 ± 800 (900–3900)	47 ± 14 (21–72)	66 ± 7 (54–78)	74 ± 9 (57–89)	23 ± 8 (10–35)	32 ± 11 (13 51)
Controls	32	5400 ± 1500 (3200–10 000)	43 ± 9 (21–61)	2300 ± 800 (1200–4900)	48 ± 13 (26–72)	67 ± 9 (46–84)	74 ± 11 (44–92)	25 ± 10 (10–47)	30 ± 11 (10–49)

Values are mean ± S D (range)
a) p = 0 02 (v controls)

TABLE 2 *Active E and Total E–RFC Tests Performed with E from 3 Different Sheep*

		Active E–RFC %				Total E–RFC %		
Active MS		A	B	C		A	B	C
Patient no	1	61	58	68		81	70	49
	2	56	53	57		73	60	57
	3	55	60	56		n d	n d	n d
	4	23	64	61		64	73	57
	5	40	44	33		62	71	62
	6	60	61	59		69	76	65
	7	70	62	69		65	80	62
	8	n d	n d	n d		82	64	58
	9	n d	n d	n d		70	61	44
	(n = 7)	52 ± 16	57 ± 7[a]	58 ± 12	(n = 8)	71 ± 8[b]	69 ± 7[c]	57 ± 7[bcde]
		(23–70)	(44–64)	(33–69)		(62–82)	(60–80)	(44–65)
Stable MS	(n = 6)	44 ± 13	42 ± 9[a]	47 ± 13	(n = 8)	64 ± 11	62 ± 7	67 ± 6[e]
		(26–67)	(26–50)	(29–66)		(48–82)	(51–72)	(54–75)
Controls	(n = 11)	46 ± 16	52 ± 15	50 ± 12	(n = 16)	68 ± 7[f]	66 ± 9	64 ± 6[df]
		(18–73)	(29–73)	(34–66)		(58–82)	(46–80)	(55–76)

Values are mean ± S D (range)
a) p = 0 008 (active MS v stable MS)
b) p = 0 03 (sheep A v sheep C)
c) p = 0 002 (sheep B v sheep C)
d) p = 0 02 (active MS v controls)
e) p = 0 02 (active MS v stable MS)
f) p = 0 04 (sheep A v sheep C)

= 0 08) for this group As this was somewhat in contrast to our previous results we decided to extend the investigation to include E–RFC tests with E from 3 different sheep When the active E–RFC and the total E–RFC tests were performed with E from 3 sheep a number of significant differences were observed (Table 2) In the active E–RFC test patients with active MS had significantly higher levels of RFC than patients with stable disease only when E from sheep B were used In the E–RFC test patients with active disease had a decreased percentage of RFC compared to patients with stable MS and controls only when tested with E from sheep C Accordingly E–RFC levels in patients with active MS were significantly lower when blood from sheep C was used rather than blood from A or B Seven out of 8 patients had lower E–RFC levels when tested with sheep C than with the other two sheep one had lower E–RFC counts with sheep A and C than with sheep B

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exacerbations However *Nordal & Frøland* (24) found the E–RFC percentage to be normal and the concentration per mm³ to be in fact increased both in patients with recent MS and those with old MS *Santoli et al* (32) found similar results but included only 2 patients with active disease while *Sagar & Allonby* (31) found T cell percentages to be low in all phases except the acute phase with low absolute T cell concentrations in all phases

A normal percentage of E–RFC in MS patients has also been found by many (2 4 5 6 8 12 14 26 28 36) but not by all (19 29 30) investigators who have not explicitly included patients with active disease

Our findings indicate that the demonstration of a decreased percentage of E–RFC in blood from patients with active MS may be dependant on an inherent quality of the E from the individual sheep *Steel et al* (35) examining healthy adults found the result of the test strongly dependant on the origin of the sheep erythrocytes while *Mendes et al* (21) found no differences between the sheep tested Using the active E test *Wybran & Fudenberg* (38) found decreased levels in cancer patients with E from 1 of 6 sheep tested while *Brain et al* (3) using an »early« RFC test did not find any differences between E from several sheep tested However no previous investigations of E–RFC with E from different sheep seem to have included both patients and healthy controls Obviously other diseases should also be examined

Active E–RFC counts have been comparatively sparingly performed in MS nevertheless conflicting results have been obtained increased levels (19) normal levels (4 6 8 11 18 26) and decreased levels (10 14) Our results are in accordance with the majority but in our opinion the possibility remains that more extensive studies with E from several different sheep might reveal differences in active E–RFC as well as for total E–RFC counts The percentages of E_{AET} RFC were very similar in the 3 groups studied So were the EAC–RFC and EA–RFC percentages indicating that there is no change in the levels of complement or Fc receptor bearing lymphocytes during MS This is in accordance with studies by *Kateley & Bazzell* (14) *Lamoureux et al* (15) *Lisak et al* (patients with stable disease) (17) *Platz et al* (28) *Nordal & Frøland* (24) and *Mar et al* (18) However increased levels of EAC–RFC have been found by *Lisak et al* (in active disease) (17) *Massoud et al* (19) *Oger et al* (27) and *Silberberg et al* (33) while *Nowak & Wajgt* (25) found a decrease in EAC–RFC The same authors also found the EA–RFC percentage to be decreased in contrast to *Mar et al* (18) who found normal values

The results of most investigations indicate a disturbance of the T lymphocyte compartment in MS but the results of the active and total E–RFC tests have varied greatly One explanation for the variation in results may be that the E–RFC decrease in active MS can only be demonstrated with E from certain sheep

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Acta path microbiol scand Sect C 88 299–302 1980

COMMON VARIABLE IMMUNODEFICIENCY AND PURINE NUCLEOTIDASE AND NUCLEOSIDE PHOSPHORYLASE DEFICIENCY

A Case Report

POUL AABEL ØSTERGAARD AAGE DEDING JAN ERIKSEN and JOHANNES MEJER

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The results of immunological and purine enzyme investigations in an adult male patient with common variable immunodeficiency recurrent lymph node granuloma and splenomegaly are presented Serum immunoglobulins were present in trace amounts only and a progressive loss of Ig bearing peripheral lymphocytes were demonstrated Furthermore the mitogenic responses to PHA ConA and PWM were markedly reduced and the ratio of T_m/T_g cells was decreased Finally a combined deficiency of lymphocyte purine 5 nucleotidase and nucleoside phosphorylase was demonstrated in the patient

Key words Common variable immunodeficiency lymph node granuloma splenomegaly purine 5 nucleotidase deficiency nucleoside phosphorylase deficiency

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Received 27 iii 80 Accepted 20 vi 80

Common variable immunodeficiency (CVI) is characterized by impairment of both humoral and cell mediated immunity and the syndrome is highly variable in severity age of onset and patterns of immunoglobulin and T-cell deficiency (*Geha et al* 1974) Furthermore heterogeneity at the biochemical level has been reported as 10 to 25 per cent of the patients have different purine enzyme deficiencies (*Gibblet et al* 1975)

We wish to report on a 34 year old male patient with CVI and decreased lymphocyte purine 5 nucleotidase (5 ND)- and nucleoside phosphorylase (PNP) In addition the patient had recurring granuloma of his lymph nodes and splenomegaly

MATERIAL AND METHODS

Case Report

A 34 year old male patient with a history of recurrent and chronic sino pulmonay infections over the past 20

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A 34 year old male patient with a history of recurrent and chronic sino-pulmonary infections over the past 20 years was in 1978 referred to the Medical Department B Aalborg Hospital for severe pneumonia In 1957 at the age of 14 years the patient had been treated at the lung clinic for recurrent bronchitis Sputa for acid fast organisms were negative but as his father had been treated earlier for lung tuberculosis and the patient was found to be Mantoux negative he received a BCG vaccination Three months later he was found to be Mantoux positive (8 mm)

In 1958 and 1961 respectively the patient had slow growing tumors in his right abdomen and in his right groin removed Histological examinations of the tumors revealed enlarged lymph nodes with expansion of the pulps and prominent reactive germinal centres A considerable proliferation of reticulum cells was seen and in addition sarcoid like granuloma were found scattered in the tissue Acid fast bacilla were not looked for In 1958 low normal serum gammaglobulin concentrations were observed (7 g/l) and Mantoux I as well as Mantoux II were negative Finally a firm moderately enlarged spleen was found

In 1963 the patient was admitted to another hospital

for severe bronchopneumonia. He recovered slowly and an X-ray film of his abdomen showed a spleen extending to the III lumbar vertebra. The patient's gammaglobulin concentration was low (5.6 g/l) and peripheral blood smears revealed a profound lymphopenia.

At the present admission the patient was very ill with pansinusitis and bilateral pneumonia. Multiple sputa revealed encapsulated non-b *Haemophilus influenzae* but acid-fast organisms were not demonstrated. When treated with ampicillin and Gammavenin he recovered slowly. Scintiographic examination showed a greatly enlarged spleen with an estimated weight of 1100 g.

After his discharge he was treated with bi-monthly intramuscular injections of gammaglobulin and since then he has had no further hospitalization. However, he has experienced many episodes of recurrent sinusitis and bronchitis.

Immunological Investigations

Serum Ig assays were performed by an electroimmunotechnique (*Østergaard* 1976). The lower limits of detection of IgG, IgA and IgM in serum were 0.1, 0.01 and 0.02 g/l, respectively. Serum IgE was measured with a paper radio-immuno-sorbent test (PRIST, Pharmacia, Copenhagen). The lower limit of detection was 5 IU/ml. *Assays for E-rosette test (E-RFC), EAC (erythrocyte-antibody-complement) rosette forming cells (RFC) and IgG-, IgA-, IgM- and IgE-bearing peripheral lymphocytes* have also been reported elsewhere (*Østergaard & Eriksen* 1977). In brief, lymphocytes were isolated on Ficoll-Isopaque and E-RFC were determined by the method of *Jondal et al.* (1972). EAC-RFC were studied by the method of *Mackler et al.* (1976). Lymphocytes with three or more sheep red blood cells attached to the surface were counted as rosettes.

For Ig-bearing cells the procedure was essentially that of *Winchester & Fu* (1976). Incubation with the FITC-conjugated antisera was carried out at 4 °C. Cells with a bright fluorescing semicircular ring were counted under a fluorescence microscope.

For the studies of *phytohaemagglutinin (PHA), concanavalin A (Con A) and pokeweed mitogen (PWM) stimulated lymphocyte cultures*, 3×10^{5} cells were cultured in round-bottomed microculture plates with or without PHA, Con A and PWM. 5 and 1 microgram PHA, 12.5 and 6.25 microgram Con A, and 20 and 10 microgram PWM were used per culture and the cultures were incubated in humidified air with 5 per cent CO_2 for 72 hours. Eighteen hours before stopping cultures, 0.12 microCi ^{14}C-thymidine was added to each well. The

performed by the method of *Moretta et al.* [illegible] In brief, T cells forming rosettes with sheep red blood cells were isolated from non-adherent mononuclear cells by density gradient centrifugation. T suppressor (T_G) and T helper (T_M) cells were counted after formation of rosettes with ox erythrocytes coated with subagglutinating amounts of rabbit IgG or IgM, respectively.

Purine Enzyme Assays

Purine enzymes were measured by the *method of Meyer & Nygaard* (1980). Mononuclear cells and granulocytes were harvested from peripheral blood after separation on a Ficoll-Isopaque gradient. The proportion of lymphocytes in the separated *mononuclear layer* exceeded 95 per cent, the rest being monocytes. Some samples contained a small *proportion of granulocytes* (less than 2 per cent).

The separated lymphocytes were *frozen and thawed* by resuspension in 0.1 M tris-HCl, pH 8.1. The cells were *disrupted by sonification and centrifugated at 8000 g* for 5 min and the supernatants were used for enzyme *analysis. Purine nucleoside phosphorylase and adenosine* deaminase (ADA) were determined spectrophotometrically *and 5'-nucleotidase* was measured in a radiochemical assay (*Meyer & Nygaard* 1980). Activities are expressed as nmol substrate converted per hour per mg *protein at 37 °C. Protein was determined according to Lowry et al.* (1951).

TABLE 1. *Immunologic Data of the Patient*

	on admission	one year later	20 adult controls (median)
Total number of lymphocytes ($\times 10^{6}$/l)	3.3	2.4	4.2
E-RFC	90	82	74.5
Ig-bearing cells (%)			
IgG	0	0	2.5
IgA	0	0	1.0
IgM	3	0	9.5
IgE	0	0	0.5
EAC-RFC (%)	2	4	18
Mitogenic responses (counts/min)			
PHA	4×10^{3}	2×10^{3}	2.5×10^{4}
Con A	ND	3×10^{3}	2.0×10^{4}
PWM	1×10^{3}	1.5×10^{3}	1.5×10^{4}
Titer anti-B (blood group A)	<1:8	1:8	1:64
Serum			
IgG (g/l)	<0.1	0.1	11.5
IgA (g/l)	0.02	<0.01	1.8
IgM (g/l)	0.05	<0.02	1.4
IgE (IU/ml)	5	<5	64
T_M (%)	ND	32	61
T_G (%)	ND	17	12
Ratio T_M/T_G	ND	1.9	5.15

ND: Not Done

TABLE 2 *Adenosine Deaminase (ADA) Purine Nucleoside Phosphorylase (PNP) and 5 nucleotidase (5 ND) Values (nm les/mg Protein/Hour) in the Patient and in Eighteen Donors with Normal Leucocyte Differential Counts and Hemoglobin (27–60 Years of Age)*

	The patient		Controls	
Enzyme	on admission	one year later	median	range
ADA	393	385	395	170–611
PNP	900	1913	6084	2916–6642
5 ND	10	21	61	28–158

RESULTS

The immunologic data of the patient and 20 healthy agerelated adults are shown in Table 1 The number of circulating E RFC was increased whereas the number of EAC RBC was very low in the patient On admission only 3 per cent IgM bearing cells was found and one year later Ig bearing cells were not seen in the blood of the patient In addition *in vitro* mitogenic responses to PHA Con A and PWM were very low and serum immunoglobulins were present in trace amounts only Finally a low number of T_M and an increased number of T_G resulting in a decreased ratio T helper/T-suppressor cells were shown

The results of estimations of purine enzymes in the patient and in healthy controls are presented in Table 2 ADA concentrations were normal in the patient whereas invariable low PNP and 5 ND levels were found

DISCUSSION

The patient in the present study is considered to have common variable immunodeficiency (CVI) as defined by the WHO classification of primary immunodeficiencies (*Fudenberg et al* 1971) However the heterogeneity of this syndrome is considerable and demands further clarification (*Geha et al* 1974 *Waldmann et al* 1974 *Dorsch et al* 1978)

ADA deficiency has been found to be associated with an autosomal recessive form of severe combined immunodeficiency (SCID) which is characterized by lymphopenia defective proliferation of lymphocytes in response to mitogens and antigens defective antibody synthesis and death at an early age due to infections (*Gibblet et al* 1972 *Meuwissen et al* 1975)

Johnson et al (1977) originally described 11 adult patients with CVI Almost all of these patients had very low 5 ND activity and the authors suggested that 5 ND activity is necessary for normal lymphocyte functions which may be related to its role in facilitating newly formed purines across cell membranes by converting them from nucleotides to nucleosides

In 1975 *Gibblet et al* published a report on a 5 year old girl with PNP deficiency However their patient as well as similar patients reported by *Hamed et al* (1977) and *Stoop et al* (1977) had normal B-cell function Furthermore *Carapello de* [illegible]

normal serum Ig levels decreased considerably and the authors suggested that the terminal defect of B cell function in their patient was the consequence of the PNP deficiency observed This suggestion is not supported by the report from *Borgers et al* (1977) that PNP activity is not present in normal B-cells

A combined deficiency of lymphocyte PNP and 5 ND was found in the present patient and to our knowledge this combined purine enzyme defect has not been reported in other patients with CVI In our patient loss of B and T-cell activity seem to have occurred when he was 14 years old as evidenced by the disappearance of an earlier positive Mantoux reaction and a decrease in serum immunoglobulin levels at that age These findings may support the theory that the enzyme defects observed in the patient had a progressive deleterious effect on B as well as on T-cell activities

On the other hand the combined PNP and 5 ND deficiency may predominantly involve T lymphocytes with loss of T cell helper activity resulting in a progressive loss of B-cell functions However B but not T-cells were absent in the patient which suggests that B-cells may be more vulnerable to the enzyme defects found

The sarcoid like granuloma of lymph nodes and splenomegaly in our patient resemble in that respect a SCID patient reported by *Edelstein et al* (1978)

and it is apparant that granuloma indistinguisable from sarcoidosis can be found in SCID patients (*Brunsky & Dunn 1965*) The pathogenesis of these lesions is unclear Our patient received a BCG-vaccination one year before the sarcoid like granuloma were detected in his lymph nodes and although speculative these lesions may represent a deficient immune response to chronic antigenic stimulation It is noteworthy that splenomegaly has been reported in more than 50 per cent of patients with idiopathic late onset immunodeficiency (*Hermans et al 1976*)

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EFFECTS OF POLYCATIONIC COMPOUNDS ON MITOGEN STIMULATION

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Key words Polycationic compounds mitogen stimulation

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The activation of lymphocytes by mitogens *in vitro* has attracted much attention and been a matter of extensive investigation for several years In spite of this little is known of the cellular processes involved in the inductive phase of mitogen reactions Several drugs have been introduced (3) to assist in analyzing these reactions and it appears that some of these are polycations Novogrodsky (10) showed that reduction of the negative cell surface charge resulted in potentiation of rat lymphocyte responses to phytomitogens We report in the present paper results of experiments to analyze the effects of simple polycationic compounds on human lymphocytes responding to mitogens

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Medium 199 or RPMI 1640 (Gibco N Y) containing 100 IU penicillin/ml was supplemented with heat inactivated normal human serum for standard culture medium Phytohemagglutinin P (PHA P Difco Detroit Mich) concanavalin A (conA Pharmacia Uppsala) and pokeweed mitogen (PWM Difco) were mitogens used Stock solutions were prepared in Dulbecco s phosphate buffered saline (PBS) at concentrations of from 1–4 mg mitogen / ml and stored for up to 2 months at 4 °C

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Lymphocyte Cultures

Heparinized or glass bead defibrinated human peripheral venous blood was obtained from healthy male and female volunteers aged 22 to 43 A total of 12 unrelated persons were used Mononuclear cells were obtained by centrifugation on Ficoll Isopaque The cell suspensions were washed twice in medium counted and adjusted to 1×10^6 viable cells per ml culture medium Duplicate or triplicate cultures were set up in microtiter plates (Nunclon Nunc, Roskilde Denmark) Each culture normally contained $1-2 \times 10^5$ mononuclear cells in a volume of 0 2 ml Mitogens and polycations were added in µl quantities from the stock solution or dilutions from these Cultures were maintained in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C Before harvest (18 h) 0 02 µCi of ^{14}C-thymidine (Amersham U K) (specific activity 61 mCi/mM) was added to each well The cultures were harvested as described elsewhere (6) The results are presented as mean cpm values which normally differed from the individual ones by less than 10%

Separation of subpopulations was performed as described previously (5 6) Cell survival in cultures was determined by counting the number of cells excluding trypan blue

RESULTS

The Effects of Polycations on Lymphocyte Responses to Mitogens

The four polycations tested repeatedly were protamine sulphate polylysine DEAE-dextran and MHSA In essence they affected mitogen responses similarly inhibition at high, augmentation at intermediate and no effect at lower concentrations The influence on different doses of mitogens and the amount of serum present in the culture medium was also studied Fig 1 shows the results of typical experiments using PHA and conA as mitogens A range of mitogen concentrations was added to lymphocytes in cultures with 2% or 15% serum, and one or several concentrations of polycations were added to the culture immediately thereafter Several conclusions emerge *Higher concentrations of polycations* were inhibitory at all mitogen concentrations at conditions with low serum % whereas slightly lower concentrations of polycations potentiated the response at the sub-optimal mitogen concentrations but inhibited at the optimal and *supraoptimal* concentrations of conA and PHA Lower polycation concentrations potentiated mitogen responses at *all concentration* At the *higher serum concentration* higher mitogen concentrations were needed to get the optimal blastogeneic responses which were quantitatively comparable in cpm to that at the lower serum % The higher polycation concentrations were now found stimulatory or less inhibitory, compared to conditions with

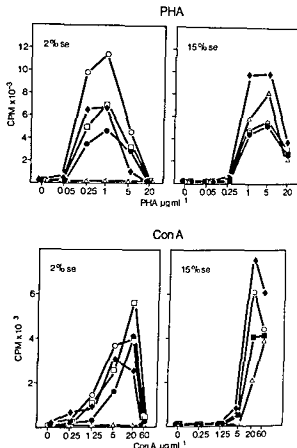


Fig 1 PHA and conA dose response curves at the indicated serum concentrations in the medium The signatures represent

- ● no polycation added (control)
- △ MHSA 25 µg/ml^{-1} added
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- O MHSA 1 µg/ml^{-1} added
- □ poly D lysine 1 µg/ml^{-1}

2% se, whereas the former supraoptimal polycation concentrations were now optimal The lower polycation concentration had no effect in medium containing 15%se

Fig 2 shows that the enhancing effect of

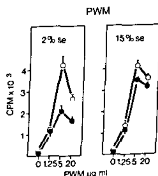


Fig 2 PWM dose response curves
- ● no polycation added
- O polylysine 1 µg/ml^{-1} added

M



Fig 3 Increasing amounts of polylysine added to mitogen stimulated cultures Two separate experiments are shown
● PHA 1 25 µg/ml⁻¹
O con A 2 5 µg/ml⁻¹
□ con A 10 µg/ml⁻¹

TABLE 1 *Per Cent Increase in Thymidine Uptake by Pretreatment with Polycation*

Expt No	Mitogen	Pretreatment 1 h	2 h	24 h	Total period
1	PHA	0	6 7	58 0	306 2
	ConA	0	16 9	60 3	179 1
2	PHA	–	83 5	111 1	293 8
	ConA	–	80 6	158 0	242 0
3	PHA	0	17 5	36 2	138 7
	ConA	0	25 0	23 6	70 8
4	PHA	0	18 3	80 6	109 3
	ConA	0	60 5	114 0	271 9

polycations on PHA and conA stimulation also holds true for PWM responses although the effect on this mitogen is less pronounced at the higher se % in the cultures

Polycations in concentrations that were augmentative for mitogen responses were not found to be mitogenic in cultures without mitogen

The inhibitory effect of higher amounts of polycations at supraoptimal mitogen concentrations was found to be particularly pronounced when con A was used (Fig 3)

The following experiments were performed in an attempt to analyze the effects observed The kinetics in the mitogen stimulated cultures was studied by harvesting at different days (Fig 4) The augmentative and inhibitory properties of polycations (not shown) were present at all times and altered kinetics was excluded as an explanation for the effect

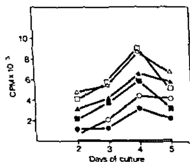


Fig 4 The kinetics of mitogen stimulation responses Closed symbols refer to cultures without polycation open symbol to those where 1 µg/ml⁻¹ polylysine is added
O ● PWM 5 µg/ml⁻¹
△ ▲ PHA 1 25 µg/ml⁻¹
□ ■ con A 5 µg/ml⁻¹
nonstimulated cultures

The Effects of Polycations on Cell Survival in Culture

Lymphocytes were cultured in the presence of polycations at concentrations producing augmented and inhibited conA response It was found that at concentrations causing inhibition of responses cell survival was reduced in particular in cultures also containing supraoptimal mitogen concentrations whereas doses causing augmented responses were either slightly toxic or had no demonstrable effect on cell survival

Pretreatment of cells Pretreatment of cells with »augmentative« amounts of polycations which were subsequently washed away was tried for 1 2 and 24 h prior to culturing with mitogens (Table 1) One hour of pretreatment had no demonstrable effect whereas pretreatment for longer had a consistently

TABLE 2 *Addition of Protamine Sulphate at Different Days of Culture Per Cent Increase (+) or Decrease (–) in Thymidine Uptake*

Mitogen	Days of cultures 0	1	2	3
PHA	+312	+109	+ 3	– 11
ConA	+160	+108	+16	–0 9

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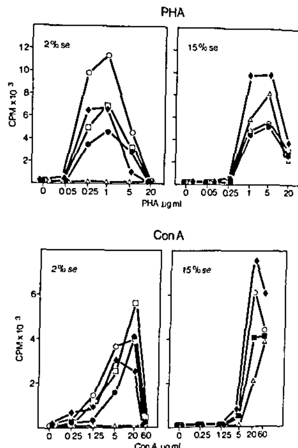


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The four polycations tested repeatedly were protamine sulphate, polylysine, DEAE dextran and MHSA. In essence they affected mitogen responses similarly: inhibition at high, augmentation at intermediate and no effect at lower concentrations. The influence on different doses of mitogens and the amount of serum present in the culture medium was also studied. Fig. 1 shows the results of typical experiments using PHA and conA as mitogens. A range of mitogen concentrations was added to lymphocytes in cultures with 2% or 15% serum and one or several concentrations of polycations were added to the culture immediately thereafter. Several conclusions emerge. *Higher concentrations of polycations* were inhibitory at all mitogen concentrations at conditions with low serum %, whereas slightly lower concentrations of polycations potentiated the response at the sub-optimal mitogen concentrations but inhibited at the optimal and *supraoptimal* concentrations of conA and PHA. Lower polycation concentrations potentiated *mitogen responses at all concentration*. At the *higher serum concentration* higher mitogen concentrations were needed to get the optimal blastogeneic responses which were quantitatively comparable in cpm to that at the lower serum %. The higher polycation concentrations were now found stimulatory or less inhibitory compared to conditions with 2% se, whereas the former supraoptimal polycation concentrations were now optimal. The lower polycation concentration had no effect in medium containing 15% se.

Fig. 2 shows that the enhancing effect of

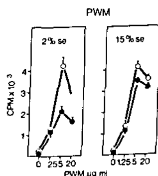


Fig. 2. PWM dose response curves.
- ● no polycation added
- ○ polylysine 1 μg/ml⁻¹ added

Mean of triplicate cultures + 1 SD are shown.

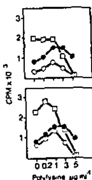


Fig 3 Increasing amounts of polylysine added to mitogen stimulated cultures Two separate experiments are shown
● PHA 1 25 µg/ml⁻¹
O con A 2 5 µg/ml⁻¹
□ con A 10 µg/ml⁻¹

polycations on PHA and conA stimulation also holds true for PWM responses although the effect on this mitogen is less pronounced at the higher se % in the cultures

Polycations in concentrations that were augmentative for mitogen responses were not found to be mitogenic in cultures without mitogen

The inhibitory effect of higher amounts of polycations at supraoptimal mitogen concentrations was found to be particularly pronounced when con A was used (Fig 3)

The following experiments were performed in an attempt to analyze the effects observed The kinetics in the mitogen stimulated cultures was studied by harvesting at different days (Fig 4) The augmentative and inhibitory properties of polycations (not

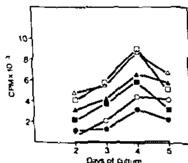


Fig 4 The kinetics of mitogen stimulation responses Closed symbols refer to cultures without polycation, open symbol to those where 1 µg/ml⁻¹ polylysine is added
O● PWM 5 µg/ml⁻¹
△▲ PHA 1 25 µg/ml⁻¹
□■ conA 5 µg/ml⁻¹

TABLE 1 *Per Cent Increase in Thymidine Uptake by Pretreatment with Polycation*

Expt No	Mitogen	Pretreatment 1 h	2 h	24 h	Total period
1	PHA	0	6 7	58 0	306 2
	ConA	0	16 9	60 3	179 1
2	PHA	–	83 5	111 1	293 8
	ConA	–	80 6	158 0	242 0
3	PHA	0	17 5	36 2	138 7
	ConA	0	25 0	23 6	70 8
4	PHA	0	18 3	80 6	109 3
	ConA	0	60 5	114 0	271 9

shown) were present at all times and altered kinetics was excluded as an explanation for the effect

The Effects of Polycations on Cell Survival in Culture

Lymphocytes were cultured in the presence of polycations at concentrations producing augmented and inhibited conA response It was found that at concentrations causing inhibition of responses cell survival was reduced in particular in cultures also containing supraoptimal mitogen concentrations whereas doses causing augmented responses were either slightly toxic or had no demonstrable effect on cell survival

Pretreatment of cells Pretreatment of cells with »augmentative« amounts of polycations which were subsequently washed away was tried for 1, 2 and 24 h prior to culturing with mitogens (Table 1) One hour of pretreatment had no demonstrable effect, whereas pretreatment for longer had a consistently enhancing effect on responses to suboptimal mitogen concentrations The effect obtained by pretreatment however was never as pronounced as that

TABLE 2 *Addition of Protamine Sulphate at Different Days of Culture Per Cent Increase (+) or Decrease (–) in Thymidine Uptake*

Mitogen	Days of cultures 0	1	2	3
PHA	+312	+109	+ 3	– 11
ConA	+160	+108	+16	–0 9

TABLE 3 *Effect on B and T Enriched Populations (Mean CPM ± SD)*

Cells responding	Polycation[a]	PHA μg/ml^{-1} 0 25	PHA μg/ml^{-1} 1 25	ConA μg/ml^{-1} 0 5	ConA μg/ml^{-1} 5
Unsep	–	8407 ± 1323	9686 ± 442	642 ± 15	3784 ± 54
	+	12326 ± 461	12805 ± 350	1287 ± 333	6396 ± 212
T	–	237 ± 2	4491 ± 214	33 ± 3	60 ± 2
	+	647 ± 217	6313 ± 144	36 ± 5	95 ± 2
T + mono	–	NT	7069 ± 263	NT	3281 ± 127
	+	NT	9336 ± 1277	NT	4929 ± 188
B	–	NT	561 ± 68	NT	257 ± 43
	+	NT	643 ± 35	NT	333 ± 35
B + mono	–	NT	975 ± 142	NT	586 ± 31
	+	NT	934 ± 63	NT	502 ± 55

[a]) MHSA was used at a concentration of 1 μg/ml^{-1}
Unseparated cells included 68 2% T cells and 11 8% monocytes
T cell fraction included 95 6 % T cell marker positive cells and <0 2% monocytes
T + mono included 95 6% T lymphocytes and 5% monocytes
B cell fraction included 0 4% T lymphocytes and 1 8% monocytes
B + mono included 0 4% T lymphocytes and 6 8% monocytes
Non stimulated cultures gave 24–93 CPM (range)

obtained by the presence of the polycation during the total culture period

Addition of polycations at different days after initiation of PHA and conA stimulated cultures showed decreasing effect from day to day (Table 2) Addition at the very latest days before or together with the thymidine pulse resulted in decreased thymidine incorporation thus excluding that the augmentory effect obtained when polycations are present during the total culture period is due to increased thymidine utilization or transport To control that the increased thymidine uptake in cultures exhibiting augmented responses to mitogens was indeed due to increased DNA synthesis cell cytofluorometry was performed in one experiment by the measurement of the relative distribution of cells in different stages of cycle DNA content was quantitated in a cytofluorophotometer subsequent to nuclear staining with ethidium bromide as described elsewhere *(13)* It was confirmed (results not shown) that the increased thymidine uptake corresponded to higher numbers of cells in the S phase

Effects on Different Mononuclear Cell Populations

Experiments were carried out to determine whether polycations act by bypassing macrophage functions or by selective support to B cells which normally respond only to a very limited extent to conA and PHA stimulation Table 3 shows the result of one such experiment The conclusion drawn was that polycation does not a) provide the macrophage help nor b) turn B cells on to respond to the mitogens c) The augmentative effect is exerted also on purified T cells

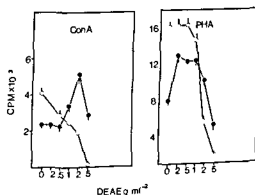


Fig 5 The effect of pretreatment of 10 ml culture medium including 2% serum with different amounts of insolubilized polycation (DEAE sephadex) The responses of conA (2 5 μg/ml^{-1}) and PHA (1 25 μg/ml^{-1}) are ● ● [illegible] m add [illegible] on

Effects on Normal Inhibitory Serum Factors

The possibility that polycations acted partly on factors present in the serum supplement in the culture medium was tested in experiments exemplified in Fig 5 Culture medium containing 2% serum was absorbed with increasing amounts of insolubilized DEAE dextran (DEAE sepharose) and subsequently assessed for supportive properties to a suboptimal concentration of conA and an optimal PHA concentration The culture medium was potentiated as far as the mitogen responses were concerned after absorption with the intermediary amounts of polycations Too heavy absorption (5 g/10 ml) reduced the supportive capacity and further addition of soluble protamine sulphate subsequent to polycation absorption had an inhibitory activity The conclusion was that apparently some but not all of the enhancing effects of polycations are due to neutralizing properties on negatively charged inhibitory proteins in serum

Effects on Cell Contact in Cultures

The number of cells responding and present in the culture well with a fixed surface area and geometry were varied by simple dilutions as well as by the addition of irradiated autologous »filler« lymphocytes The possible augmentative effects of polycations on mitogen responses were assayed under such conditions The most pronounced augmentation was observed at the lowest cell densities ($1-2 \times 10^5$ cells/culture well)

DISCUSSION

The compounds studied in the present paper are very different and unrelated chemicals that share the property of having strong positive charge Such polycationic compounds have a potential interest because they have been found to inhibit tumor cell growth *in vivo* (for review see *Larsen* (7)) *The exact* basis for this effect is not known but one of the possibilities is that an immuno-stimulatory action is exerted by polycations In the present study augmentation of lymphocyte responses to mitogens was seen within certain dose ranges The *impor*tance of this as an explanation for the *in vivo* effect outlined above naturally has to be considered with caution

Novogrodsky (10) first described the stimulatory effect of polycation *in vitro* using rat lymphocytes stimulated with phytomitogens The present study has extended this observation by testing *other* polycationic compounds by the use of human lymphocytes as responder cells and by a close analysis of the *in vitro* effects The effect of compounds like cytochalasin B which has been investigated *in vitro* in assay systems similar to that used here by several groups (3 4 14) is thought to exert a specific effect on cells Cytochalasin B however is a positively charged molecule and the *in vitro* effects as described by others are fully comparable to those described in the present investigation for simple positively charged chemicals

The effects could in principle be on the mitogens on the medium on the cells or on a combination of these and we have attempted to analyze the system under this scope Trivial explanations for the effects described like shift in kinetics better survival of cells or effect on thymidine transport have been excluded

It became clear from the dose titrations of mitogens under conditions of high or low serum content in the medium that a certain binding or inactivation by serum factors of mitogens most pronounced in the case of conA is taking place The polycation interaction in the culture system partly depends on this since doses of polycat*ions* potentiating the mitogen response at high serum concentration were inhibitory at the lower serum concentration Furthermore the addition of polycations at a fixed serum concentration shifted the mitogen dose response curve to the left again most pronounced with conA Probably polycations interact with mitogen binding serum substances thus making the mitogen available for reacting with the cells

An interaction is taking place between polycation and negatively charged serum components which normally have an inhibitory effect on the responding cells (8 2 9 1) Indication for this assumption came from the experiments in which the serum containing medium had been adsorbed with insolubilized polycation (Fig 5) Such medium supported mitogen induced responses better than non adsorbed medium suggesting that inhibitory substances had been removed *From the DEAE sephadex beads* used for adsorption inhibitory glycoproteins could be eluted (unpublished) If culture medium was adsorbed too extensively (>2 g DEAE sephadex *per 10 ml* medium – 2% serum) however inhibition of growth was obtained which suggests that certain amounts of the substances binding to *the DEAE* beads are necessary for cells in culture A need for certain negatively charged serum components may also explain the inhibitory and toxic action of the addition of the higher concentra[illegible]

[illegible] at lower doses of conA in that both

substances bind to glycoproteins in the medium thus leaving the cells »unprotected«

Pretreatment of cells before culture was found to potentiate the subsequent response *in vitro* to mitogens This corresponds to the observation that increased uptake of particles and compounds by cells has been shown following polycation treatment (12) The enchancing effect of lymphocyte pretreatment was however never as pronounced as the effect of having the polycation present in the full culture period but the result suggests that part of the action might be due to a direct cell surface interaction

The possibility that other lymphocyte classes than normally might be recruited to respond to the mitogens when polycations were added was tested by assaying the effects on isolated subpopulations of lymphoid cells from the blood These results excluded that polycations substitute for monocytes and that B cells are on their own becoming responsive to the T cell mitogens We have not been able to decide whether interaction between different classes of cells during the normal response of unseparated cells to mitogen is influenced by polycations The results of the experiments with different cell densities in the cultures might be taken as an indication of altered cell to cell interaction Cell to cell contact is influenced by surface charges and it is conceivable that cultures with relatively low cell numbers might benefit from decrease in negative charge easing mutual cell contact

In conclusion the effects of polycationic compounds on lymphocyte responses to phytomitogens *in vitro* are very complex multihit phenomena One is action on mitogen binding serum factors another is action on »immunosuppressive« serum components a third are effects directly by interaction with the cell surface

The technical assistance by Mrs *I Sørensen* and Mr *A Muller* is sincerely acknowledged

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IgG AND OTHER PROTEINS ASSOCIATED WITH HUMAN CARCINOMAS AND CANCER-FREE TISSUE FROM THE SAME ORGANS

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Wesenberg F & Tonder O IgG and other proteins associated with human carcinomas and cancer free tissue from the same organs Acta path microbiol scand Sect C 88 309-312 1980

Eluates of different tissues were prepared at 56 °C using a continuous flow technique More IgG and other serum proteins were found in liver tissue from patients with non malignant diseases than in liver tissue from healthy control individuals Cancer free liver tissue from patients with carcinomas was similar to that of patients with non malignant diseases but liver metastases contained twice the amount of IgG No differences were found between non perfused carcinomas of the kidney and cancer free renal tissue whereas more IgG was presen ...
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Key words Human carcinomas IgG

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Received 22 i 80 Accepted 1 ix 80

We have previously shown that eluates of malignant tissues contain more IgG and other proteins than do eluates of organs from healthy individuals and that no IgG was found in eluates of normal liver (8 10) Since *Romsdahl & Cox* (3) reported that eluates of control liver tissue from patients with sarcoma contained IgG and several other proteins similar to the eluates of the sarcoma tissue the question therefore arose whether there were any qualitative or quantitative differences between eluates of 1) livers from healthy individuals compared to livers from patients with malignant and non malignant diseases and 2) liver metastases compared to cancer free liver tissue from the same patient As we have observed that renal tissue contains more tissue bound proteins than other normal tissues (8 10) we have also compared eluates of carcinoma of the kidney and of cancer free tissue from the same kidney

MATERIALS AND METHODS

Tissue

The following tissues were obtained at autopsy from the Department of Pathology Well-defined liver metastases and cancer free liver tissue from 7 patients with carcinoma of the colon and from 3 patients with carcinoma of the stomach liver specimens from 8 patients with non malignant disease including severe infections (septicaemia bronchopneumonia peritonitis) ulcerative colitis regional ileitis and ankylosing spondylitis Liver specimens from 8 patients with acute heart failure and no other signs of disease served as normal controls All specimens were obtained 8-20 h after death ...
from th...
perfuse...
remova ... tissue was carefully removed and 5 x 5 x 5 mm tissue blocks were snap frozen in liquid nitrogen and mounted for the preparation of cryostat tissue sections The remainder of the tissue was either used immediately or stored at -25 °C Histological

examination was routinely performed at the Department of Pathology

Sera

Sera were obtained from 14 of the autopsies and preoperatively from all the patients at the Department of Surgery. Pooled human serum (PHS) was prepared from 20 blood donors. Isolated IgG was purchased from A/B Kabi, Stockholm.

Antisera

Antiserum to PHS (anti PHS) was raised in rabbits by subcutaneous injections of PHS mixed with Freund's adjuvant. For the first injection complete adjuvant was used and for further injections incomplete.

Antisera to human α_1 foetoprotein, haptoglobin, α_2 macroglobulin, α_1 antichymotrypsin, α_1 antitrypsin, fibrinogen, β_2 lipoprotein, C3/C3c (β_1C β_1A globulin), C4 (β_1E globulin), C1 inactivator (C1 INH), C1q, IgG, IgM and IgD were purchased from Behringwerke AG, Marburg Lahn, West Germany. Antisera to human IgA, carcinoembryonic antigen (CEA), albumin and β_2 microglobulin as well as an antiserum to human gamma, kappa and lambda chains were purchased from DAKO immunoglobulins, Copenhagen.

Extracts and Eluates

Extracts and eluates were prepared as described previously (10). In brief, the tissue was homogenized in phosphate buffered saline pH 7.2 (PBS) (four times wet weight) for 1 min in a Servall Omni Mixer at circa 0 °C and centrifuged at 20000 × g for 20 min. The supernatant beneath the lipid layer was collected (extract). Two to 3 grams of tissue sediment was resuspended in PBS containing 0.05 per cent Na azide, placed between glass fiber filters in a short glass column and washed in a continuous upward flow of PBS until the optical density (OD) at 260 nm of the effluent was below 0.05. After washing, a control sample of the effluent was collected. The column was then submerged in a waterbath at 56 °C and the tissue eluted in a continuous flow of PBS (56 °C) monitored by the OD of the effluent. Control fluids and eluates were concentrated to between 1.0 to 3.0 ml using Amicon Diaflo UM 05 filters. There was no loss of proteins during the concentration procedure as measured by spectrophotometry.

Fcγ Receptor Activity (FcRA) in Tissue Sections

FcRA in tissue sections was demonstrated as previously (7) using the microculture (hanging drop) slide technique (5). The indicator cells were sheep erythrocytes (E) sensitized with varying amounts of rabbit IgG anti E (A) expressed as agglutinating units. One agglutinating unit is defined as the amount of the highest dilution of A which agglutinates an equal amount of a 1 per cent suspension of E. The strength of the haemadsorption was rated by referring to the agglutinating units of A on EA which produced a reaction on the section.

Precipitation

The double diffusion test was performed in 1 per cent agar in PBS as previously described (9). For investigation of C1q, 0.5 per cent agarose in PBS was used. Sera, extracts or eluates were applied 2 h prior to the addition of the antiserum.

Crossed Immunoelectrophoresis (CIE)

CIE was performed as described by Weeke (6). Varying amounts of antisera, extracts or eluates were used to obtain optimal precipitation conditions. An intermediate gel containing appropriate amounts of specific antisera was used to identify the various proteins.

Quantification of IgG and Albumin

IgG and albumin in sera, extracts and eluates were quantified using single immunodiffusion plates from Behringwerke AG. The lower limit of detection was 10 mg/l which corresponded to circa 6 mg eluted IgG/kg wet tissue. The ratio of the contrations of albumin to IgG in sera, extracts and eluates was calculated. The reduction of the ratio was calculated as preciously described (8).

Statistics

Wilcoxon's two sample test was used throughout.

RESULTS

Albumin was the only protein detected in all eluates. Significantly more IgG was eluted from liver tissue from patients with non malignant diseases than from healthy control individuals ($p = 0.05$) (A and B, Table 1). There was no significant difference in the eluted amount of IgG between liver tissue from patients with non malignant and with malignant disease ($p = 0.20$) (B and C, Table 1) but more eluates of the latter also contained IgA, haptoglobin, α_1 antitrypsin and α_1 antichymotrypsin. The eluates which showed the highest concentration of albumin also contained most of these additional proteins. In eluates of cancer tissue from 9 of the 10 patients twice as much IgG or more was obtained than from their liver tissue. From one patient a similar amount of IgG was eluted from the two types of tissue. For the group examined the difference between the two types of tissue was highly significant ($p = 0.01$) (C and C², Table 1). More of the eluates of the cancers contained IgA and fewer contained haptoglobin when compared to the eluates of the cancer free liver tissue.

Using non perfused kidneys the same amount of IgG was eluted from cancer free renal tissue and from cancer tissue. Several other proteins were also present, but no differences were found between the two types of eluates (D and D², Table 1). After perfusion fewer proteins were detected in the eluates (E and E², Table 1). In addition the amount of albumin was significantly lower in eluates both from the cancer of the kidney (D² and E²) ($p = 0.02$) and from the cancer free renal tissue (D and E) ($p < 0.01$) as was the amount of IgG in eluates from the cancer free renal tissue ($p = 0.03$). However, perfusion did not influence the amount of IgG eluted from the cancer tissue.

TABLE 1 *IgG and Other Serum Proteins in Eluates of Various Normal and Malignant Tissues*

Tissue	Total no	Eluted IgG[1]	Number of eluates containing: IgG	IgA	Haptoglobin	α_1 antitrypsin	α_1 antichymotrypsin	Albumin	Eluted albumin[1]
Normal liver	8	<6 (<6–10)[a]	2	0	2	0	0	8	22 5 (10– 55)[a]
Non malignant liver[3]	8	17 5 (<6–27)[b]	5	2	5	5	2	8	55 (35– 90)[b]
Cancer free liver tissue[4]	10	19 (<6–45)[b]	9	3	9	8	8	10	60 (35– 95)[b]
Liver metastases	10	36 (20–85)[F]	10	7	4	9	9	10	55 (25 85)[b]
Cancer free renal tissue	5	40 (1?–80)[r]	5	3	2	2	3	5	55 (45–110)[b]
Cancer of the kidney	5	40 (15–80)[F]	5	1	1	4	3	5	45 (35–105)[b]
Cancer free renal tissue perfused	6	14 (9 28)[d]	6	0	0	0	0	6	20 (10– 26)[d]
Cancer of the kidney perfused	6	36 (20–55)[F]	6	2	1	0	0	6	20 (11– 37)[d]

[1]) In mg/kg wet homogenized tissue median and range
[2]) Same letter indicates tissue from the same organ

...perscripts mean that the tissues are significantly

Since perfusion significantly reduced the amounts eluted of proteins considered non-specifically bound different washing and elution procedures using homogenized tissue were compared Two of the homogenized non perfused cancers and cancer free tissues were washed at 20 °C for 2 h before washing at 4 °C The tissue was then eluted at 20 37 45 and 56 °C successively The results were compared to those obtained when the tissue was eluted at 56 °C only Most proteins were eluted at 56 °C and similar amounts of IgG and albumin were eluted after washing at 20 °C as at 4 °C only

The ratio of albumin to IgG in extracts and eluates was calculated A similar ratio in eluates and corresponding extracts would indicate non specific binding of IgG to the tissue while a reduced ratio would indicate specific binding All eluates of the cancer of the kidney and cancer free renal tissue as well as 8 of the liver metastases showed a reduced ratio whereas only 2 of the cancer free liver tissues did The other 8 cancer free liver tissues as well as all the other liver tissues and the remaining 2 liver metastases showed a similar or increased ratio

Two of the carcinomas of the kidney were divided into the central and subcapsular areas before elution There were no differences between the eluates from these 2 parts of the tumours The results using cancer tissue obtained at autopsy and at surgery were also compared No differences were found (C[2] D[2] and E[2] Table 1)

CEA was detected in extracts and eluates of the cancers only and β_2-microglobulin only in the eluates of the cancers and in eluates of the cancer free renal tissue

The following proteins were not found in any of the eluates α_1 foetoprotein IgM IgD C1q C1 INH C3 C4 α_2-macroglobulin fibrinogen and β_1 lipoprotein

All eluates were also tested in CIE using anti PHS in the gel and various monospecific antisera in the intermediate gel Up to 8 different proteins were identified with no differences between the cancer tissue and the cancer free tissue from the same organs

FcRA was detected in sections of all carcinomas and livers but not in sections of cancer free renal tissue The amount of eluted IgG was plotted against the degree of FcRA in the corresponding tissue sections No relationship was found

DISCUSSION

The aim of the present study was to investigate whether there were quantitative or qualitative differences between 1) liver tissue from normal controls and from patients with cancer or non malignant diseases and 2) cancer tissue and cancer free tissue from the same organ For this purpose eluates from different liver tissues liver metastases carcinomas of the kidney and cancer free renal tissues were used

As previously shown (8 10) eluates of liver tissue from control individuals contained no or only small amounts of IgG and few other proteins whereas significantly more IgG and other proteins were associated with the liver tissue from patients with various carcinomas However this was not characteristic for such liver tissue since liver tissue from patients with non malignant diseases showed similar results

The spectrum of proteins present in eluates of liver metastases and cancer free liver tissue from the

examination was routinely performed at the Department of Pathology

Sera

Sera were obtained from 14 of the autopsies and preoperatively from all the patients at the Department of Surgery Pooled human serum (PHS) was prepared from 20 blood donors Isolated IgG was purchased from A/B Kabi Stockholm

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Antisera to human α_1 foetoprotein haptoglobin α_2 *macroglobulin* α_1 *antichymotrypsin* α_1 *antitrypsin fi* brinogen β_2 lipoprotein C3/C3c (β_1C β_1A globulin) C4 (β_1E globulin) Cl inactivator (Cl INH) Clq IgG IgM and IgD were purchased from Behringwerke AG Marburg Lahn West Germany Antisera to human IgA carcinoembryonic antigen (CEA) albumin and β_2 microglobulin as well as an antiserum to human gamma kappa and lambda chains were purchased from DAKO immunoglobulins Copenhagen

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IgG and albumin in sera extracts and eluates were quantified using single immunodiffusion plates from Behringwerke AG The lower limit of detection was 10 mg/l which corresponded to circa 6 mg eluted IgG/kg wet tissue The ratio of the contrations of albumin to IgG in sera extracts and eluates was calculated The *reduction of the* ratio was calculated as preciously described (8)

Statistics

Wilcoxon s two sample test was used throughout

RESULTS

Albumin was the only protein detected in all eluates Significantly more IgG was eluted from liver tissue from patients with non malignant diseases than *from healthy control individuals (p = 0 05)* (A and B Table 1) There was no significant difference in the eluted amount of IgG between liver tissue from patients with non malignant and with malignant disease (p = 0 20) (B and C Table 1) but more *eluates of the latter also contained IgA haptoglobin* α_1 antitrypsin and α_1 antichymotrypsin The eluates which showed the highest concentration of albumin also contained most of these additional proteins In eluates of cancer tissue from 9 of the 10 patients *twice as much IgG or more was obtained* than from their liver tissue From one patient a similar amount of IgG was eluted from the two types of tissue For the group examined the difference between the two types of tissue was highly significant (p = 0 01) (C and C² Table 1) More of the eluates of the cancers contained IgA and fewer contained haptoglobin when compared to the eluates of the cancer free liver tissue

Using non perfused kidneys the same amount of IgG was eluted from cancer free renal tissue and from cancer tissue Several other proteins were also present but no differences were found between the two types of eluates (D and D² Table 1) After perfusion fewer proteins were detected in the eluates (E and E² Table 1) In addition the amount of albumin was significantly lower in eluates both from the cancer of the kidney (D² and E²) (p = 0 02) and from the cancer free renal tissue (D and E) ($p < 0\ 01$) as was the amount of IgG in eluates from the cancer free renal tissue (p = 0 03) How ever perfusion did not influence the amount of IgG eluted from the cancer tissue

TABLE 1 *IgG and Other Serum Proteins in Eluates of Various Normal and Malignant Tissues*

[illegible]e	Total no	Eluted IgG[1]	Number of eluates containing: IgG	IgA	Haptoglobin	α1-antitrypsin	α1-antichymotrypsin	Albumin	Eluted albumin[1]
Normal liver	8	<6 (<6–10)P	2	0	2	0	0	8	22.5 (10–55)P
Non malignant liver[3]	8	17.5 (<6–27)P	5	2	5	5	2	8	55 (35–90)P
Cancer free liver tissue[4]	10	19 (<6–45)P	9	3	9	8	8	10	60 (35–95)P
Liver metastases	10	36 (20–85)F	10	7	4	9	9	10	55 (25–85)P
Cancer free renal tissue	5	40 (12–80)F	5	3	2	2	3	5	55 (45–110)P
Cancer of the kidney	5	40 (15–80)F	5	1	1	4	3	5	45 (35–105)P
Cancer free renal tissue perfused	6	14 (9–28)M	6	0	0	0	0	6	20 (10–26)M
Cancer of the kidney perfused	6	36 (20–55)F	6	2	1	0	0	6	20 (11–37)P

[1]) In mg/kg wet homogenized tissue median and range
[2]) Same letter indicates tissue from the same organ

...perscripts mean that the tissues are significantly

Since perfusion significantly reduced the amounts eluted of proteins considered non specifically bound different washing and elution procedures using homogenized tissue were compared Two of the homogenized non perfused cancers and cancer free tissues were washed at 20 °C for 2 h before washing at 4 °C The tissue was then eluted at 20 37 45 and 56 °C successively The results were compared to those obtained when the tissue was eluted at 56 °C only Most proteins were eluted at 56 °C and similar amounts of IgG and albumin were eluted after washing at 20 °C as at 4 °C only

The ratio of albumin to IgG in extracts and eluates was calculated A similar ratio in eluates and corresponding extracts would indicate non specific binding of IgG to the tissue while a reduced ratio would indicate specific binding All eluates of the cancer of the kidney and cancer free renal tissue as well as 8 of the liver metastases showed a reduced ratio whereas only 2 of the cancer free liver tissues did The other 8 cancer free liver tissues as well as all the other liver tissues and the remaining 2 liver metastases showed a similar or increased ratio

Two of the carcinomas of the kidney were divided into the central and subcapsular areas before elution There were no differences between the eluates from these 2 parts of the tumours The results using cancer tissue obtained at autopsy and at surgery were also compared No differences were found (C[2] D[2] and E[2] Table 1)

CEA was detected in extracts and eluates of the cancers only and β_2 microglobulin only in the eluates of the cancers and in eluates of the cancer free renal tissue

The following proteins were not found in any of the eluates α_1 foetoprotein IgM IgD Clq Cl INH C3 C4 α_2-macroglobulin fibrinogen and β_2-lipoprotein

All eluates were also tested in CIE using anti PHS in the gel and various monospecific antisera in the intermediate gel Up to 8 different proteins were identified with no differences between the cancer tissue and the cancer free tissue from the same organs

FcRA was detected in sections of all carcinomas and livers but not in sections of cancer free renal tissue The amount of eluted IgG was plotted against the degree of FcRA in the corresponding tissue sections No relationship was found

DISCUSSION

The aim of the present study was to investigate whether there were quantitative or qualitative differences between 1) liver tissue from normal controls and from patients with cancer or non [illegible] carcinomas of the kidney and cancer-free renal tissues were used

As previously shown (8 10) eluates of liver tissue from control individuals contained no or only small amounts of IgG and few other proteins whereas significantly more IgG and other proteins were associated with the liver tissue from patients with various carcinomas However this was not characteristic for such liver tissue since liver tissue from patients with non malignant diseases showed similar results

The spectrum of proteins present in eluates of liver metastases and cancer free liver tissue from the

examination was routinely performed at the Department of Pathology

Sera

Sera were obtained from 14 of the autopsies and preoperatively from all the patients at the Department of Surgery. Pooled human serum (PHS) was prepared from 20 blood donors. Isolated IgG was purchased from A/B Kabi, Stockholm.

Antisera

Antiserum to PHS (anti PHS) was raised in rabbits by subcutaneous injections of PHS mixed with Freund's adjuvant. For the first injection complete adjuvant was used, and for further injections incomplete.

Antisera to human α_1 foetoprotein, haptoglobin, α_2 macroglobulin, α_1 antichymotrypsin, α_1 antitrypsin, fibrinogen, β_2 *lipoprotein*, *C3/C3c* (β_1*C*, β_1*A globulin*), C4 (β_1E globulin), Cl inactivator (Cl INH), Clq, IgG, IgM and IgD were purchased from Behringwerke AG, Marburg Lahn, West Germany. Antisera to human IgA, carcinoembryonic antigen (CEA), albumin and β_2 microglobulin as well as an antiserum to human gamma, kappa and lambda chains were purchased from DAKO immunoglobulins, Copenhagen.

Extracts and Eluates

Extracts and eluates were prepared as described previously (10). In brief, the tissue was homogenized in phosphate buffered saline pH 7.2 (PBS) (four times wet weight) for 1 min in a Servall Omni Mixer at circa 0 °C and centrifuged at $20000 \times g$ for 20 min. The supernatant beneath the lipid layer was collected (extract). Two to 3 grams of tissue sediment was resuspended in PBS containing 0.05 per cent Na azide, placed between glass fiber filters in a short glass column and washed in a continuous upward flow of PBS until the optical density *(OD) at 260 nm of the effluent was below 0.05*. After washing, a control sample of the effluent was collected. The column was then submerged in a waterbath at 56 °C and the tissue eluted in a continuous flow of PBS *(56 °C) monitored by the OD of the effluent. Control* fluids and eluates were concentrated to between 1.0 to 3.0 ml using Amicon Diaflo UM 05 filters. There was no loss of proteins during the concentration procedure as measured by spectrophotometry.

Fcγ Receptor Activity (FcRA) in Tissue Sections

FcRA in tissue sections was demonstrated as previously (7) using the microculture (hanging drop) slide technique (5). The indicator cells were sheep erythrocytes (E) sensitized with varying amounts of rabbit IgG anti E (A) expressed as agglutinating units. One agglutinating unit is defined as the amount of the highest dilution of A *which agglutinates* an equal amount of a 1 per cent suspension of E. The strength of the haemadsorption was rated by referring to the agglutinating units of A on EA which produced a reaction on *the section*.

Precipitation

The double diffusion test was performed in 1 per cent agar in PBS as previously described (9). For investigation of Clq 0.5 per cent agarose in PBS was used. Sera, *extracts or eluates were applied 2 h prior to the addition* of the antiserum.

Crossed Immunoelectrophoresis (CIE)

CIE was performed as described by *Weeke* (6). *Varying amounts* of antisera, extracts or eluates were used to obtain optimal precipitation conditions. An intermediate gel containing appropriate amounts of specific antisera was used to identify the various proteins.

Quantification of IgG and Albumin

IgG and albumin in sera, extracts and eluates were quantified using single immunodiffusion plates from Behringwerke AG. The lower limit of detection was 10 mg/l which corresponded to circa 6 mg eluted IgG/kg wet tissue. The ratio of the contrations of albumin to IgG in sera, extracts and eluates was calculated. The reduction of the ratio was calculated as preciously *described (8)*.

Statistics

Wilcoxon's two sample test was used throughout.

RESULTS

Albumin was the only protein detected in all eluates. Significantly more IgG was eluted from liver tissue from patients with non malignant diseases than from healthy control individuals ($p = 0.05$) (A and *B, Table 1). There was no significant difference in* the eluted amount of IgG between liver tissue from patients with non malignant and with malignant disease ($p - 0.20$) (B and C, Table 1) but more eluates of the latter also contained IgA, haptoglobin, α_1 *antitrypsin and* α_1 *antichymotrypsin*. The eluates which showed the highest concentration of albumin also contained most of these additional proteins. In eluates of cancer tissue from 9 of the 10 patients twice as much IgG or more was obtained than from *their liver tissue*. *From one patient a similar amount of IgG was eluted from the two types of tissue*. For the group examined the difference between the two types of tissue was highly significant ($p = 0.01$) (*C* and C^2, Table 1). More of the eluates of the cancers contained IgA and fewer contained haptoglobin when compared to the eluates of the cancer free liver tissue.

Using non perfused kidneys, the same *amount of* IgG was eluted from cancer free renal tissue and from cancer tissue. Several other proteins were also present, but no differences were found between the two types of eluates (D and D^2, Table 1). After perfusion, fewer proteins were *detected in* the eluates (E and E^2, Table 1). In addition, the amount of albumin was significantly lower in eluates both from the cancer of the kidney (D^2 and E^2) ($p = 0.02$) and from the cancer free renal tissue (D and E) ($p < 0.01$) *as was the amount* of IgG in eluates from the cancer free renal tissue ($p = 0.03$). However, perfusion did not influence the amount of IgG eluted from the cancer tissue.

TABLE I *IgG and Other Serum Proteins in Eluates of Various Normal and Malignant Tissues*

Tissue	Total no	Eluted IgG[1]	Number of eluates containing						Eluted albumin[1]
			IgG	IgA	Haptoglobin	α_1 antitrypsin	α_1 antichymotrypsin	Albumin	
Normal liver	8	<6 (<6–10)a	2	0	2	0	0	8	22.5 (10–55)a
Non malignant liver[3]	8	17.5 (<6–27)b	5	2	5	5	2	8	55 (35–90)b
Cancer free liver tissue[4]	10	19 (<6–45)b	9	3	9	8	8	10	60 (35–95)b
Liver metastases	10	36 (20–85)c	10	7	4	9	9	10	55 (23–85)b
Cancer free renal tissue	5	40 (17–80)c	5	3	2	2	3	5	55 (45–110)b
Cancer of the kidney	5	40 (15–80)c	5	1	1	4	3	5	45 (35–105)b
Cancer free renal tissue perfused	6	14 (9–28)d	6	0	0	0	0	6	20 (10–26)d
Cancer of the kidney perfused	6	36 (20–55)c	6	2	1	0	0	6	20 (11–37)d

1) In mg/1 g wet homogenized tissue median and range
2) Same letter indicates tissue from the same organ
3) Liver tissue from patients with non malignant diseases (see text)
4) Cancer free liver tissue from patients with liver metastases
a b c in Tissues A C and b c d in Tissues C E different superscripts mean that the tissues are significantly different ($p < 0.05$) (Wilcoxon s two sample test)

Since perfusion significantly reduced the amounts eluted of proteins considered non specifically bound different washing and elution procedures using homogenized tissue were compared Two of the homogenized non-perfused cancers and cancer free tissues were washed at 20 °C for 2 h before washing at 4 °C The tissue was then eluted at 20 37 45 and 56 °C successively The results were compared to those obtained when the tissue was eluted at 56 °C only Most proteins were eluted at 56 °C and similar amounts of IgG and albumin were eluted after washing at 20 °C as at 4 °C only

The ratio of albumin to IgG in extracts and eluates was calculated A similar ratio in eluates and corresponding extracts would indicate non specific binding of IgG to the tissue while a reduced ratio would indicate specific binding All eluates of the cancer of the kidney and cancer free renal tissue as well as 8 of the liver metastases showed a reduced ratio whereas only 2 of the cancer free liver tissues did The other 8 cancer free liver tissues as well as all the other liver tissues and the remaining 2 liver metastases showed a similar or increased ratio

Two of the carcinomas of the kidney were divided into the central and subcapsular areas before elution There were no differences between the eluates from these 2 parts of the tumours The results using cancer tissue obtained at autopsy and at surgery were also compared No differences were found (C^2 D^2 and E^2 Table I)

CEA was detected in extracts and eluates of the cancers only and β_2-microglobulin only in the eluates of the cancers and in eluates of the cancer free renal tissue

The following proteins were not found in any of the eluates α_1 foetoprotein IgM IgD C1q C1 INH C3 C4 α_2 macroglobulin fibrinogen and β_2-lipoprotein

All eluates were also tested in CIE using anti PHS in the gel and various monospecific antisera in the intermediate gel Up to 8 different proteins were identified with no differences between the cancer tissue and the cancer free tissue from the same organs

FcRA was detected in sections of all carcinomas and livers but not in sections of cancer free renal tissue The amount of eluted IgG was plotted against the degree of FcRA in the corresponding tissue sections No relationship was found

DISCUSSION

The aim of the present study was to investigate whether there were quantitative or qualitative differences between 1) liver tissue from normal controls and from patients with cancer or non malignant diseases and 2) cancer tissue and cancer free tissue from the same organ For this purpose eluates from different liver tissues liver metastases carcinomas of the kidney and cancer free renal tissues were used

As previously shown (8 10) eluates of liver tissue from control individuals contained no or only small amounts of IgG and few other proteins whereas significantly more IgG and other proteins were associated with the liver tissue from patients with various carcinomas However this was not characteristic for such liver tissue since liver tissue from patients with non malignant diseases showed similar results

The spectrum of proteins present in eluates of liver metastases and cancer free liver tissue from the

same organ was similar confirming the results obtained by *Romsdal & Cox* (3) They used low pH eluates of sarcoma tissue and control liver tissue from the same patients and found that except for IgA the same proteins were eluted from the two types of tissue Our results further showed that significantly more IgG was eluted from the metastases than from the cancer free liver tissue This suggested that more IgG was bound to the malignant tissue To further assess whether this IgG was non specifically attached to the tissue albumin was used as a marker (8) The results obtained by comparing the ratio of albumin to IgG in eluates and corresponding sera or extracts supported the notion that more IgG was specifically bound to the malignant tissue

To further investigate this point carcinoma of the kidney and cancer free renal tissue were studied since the kidney could be perfused after removal by surgery No differences were found using eluates of non perfused organs However, perfusion removed some of the IgG from the cancer free renal tissue whereas no IgG was removed by perfusion of the malignant tissue Accordingly most of the IgG associated with 19 of the 21 tumours appeared to be specifically bound either to antigens or to Fcγ receptors (FcR) In contrast most of the IgG associated with the cancer free tissues was apparently non specifically bound

No definite evidence was obtained for IgG bound to FcR in cancer tissue extending previous results (7) This may indicate that the IgG is bound to antigens The antigens need not be tumour antigens since auto antibodies are present in sera of cancer patients (2) and since some of the IgG is specifically bound to cancer free renal tissue where no FcRA could be demonstrated Our results differ from those presented by *Wood et al* (11) They claim that a significant amount of Ig is bound to FcR in tumours from the central nervous system This discrepancy can be due to the differing types of tumours employed

Perfusion of the kidney removed most of the proteins other than Ig from the tissue showing that these proteins were non specifically bound However with the use of non perfused tissues most of these proteins were eluted at 56 °C indicating that non specifically bound proteins were comparatively firmly attached to the tissue Evidently thourough washing of tissues at 37 °C does not remove all non specifically bound IgG as claimed by others (11) The best way to remove the non specifically bound proteins seems to be perfusion of the organs immediately after removal

The same amount of IgG was eluted from the cancer tissues obtained at autopsy and at surgery showing that tumours obtained at autopsy can be used to study tumour associated IgG The results obtained using the central and the subcapsular part of the malignant tissue suggested a uniform distribution of proteins in the tissue

The presence of β_2 microglobulin in eluates of malignant tissue is of interest Increased levels of β_2 microglobulin are found in sera of patients with cancer (1) and it has been associated with tumour antigens (4)

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TISSUE REACTIVITY OF IgG ELUTED FROM HUMAN CARCINOMAS

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Mixed haemagglutination with tissue sections was used to study the tissue reactivity of IgG eluted from human carcinomas IgG eluted from 21 of 29 tumours bound to the autologous tissue The binding was mediated through the Fab portion and the bound IgG had an intact Fc portion Most eluted IgG bound to the autologous tissue but binding was also seen to other carcinomas of the same type as well as to other types In addition the IgG bound to several cancer free tissues Accordingly the eluates of the 21 tumours contained IgG with a broad tissue reactivity The eluates of a) the remaining 8 tumours b)normal tissue and c) liver tissue from patients with non malignant diseases contained IgG which did not bind to any tissue The IgG associated with these tissues was probably non specifically bound or bound to receptors *in vivo*

Key words Human carcinomas eluted IgG tissue reactivity

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Antibodies to human tumours have been detected in the serum of the tumour bearing patients (for review see ref 4) This does not necessarily mean that the antibodies are present on the tumours *in situ* (for review see ref 18) However *Gupta & Morton* (3) reported evidence for *in vivo* binding of specific antibodies to human melanoma *Romsdahl & Cox* (11) showed that Ig eluted from human sarcom... [illegible]

tissues men... [illegible]

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o ... parts of the IgG seem to be non-specifically bound to the tissue IgG was also eluted from cancer free tissue from the same organs (17) In the present work the tissue reactivity of IgG eluted from human carcinoma and from cancer free tissues was studied Evidence was obtained that the IgG eluted from the carcinomas bound to both autologous and homologous tissues as well as to cancer free tissues

MATERIALS AND METHODS

Tissue

Specimens were from the same tissues as were used in previous studies having been stored at –25 °C (16 17) Metastases from 19 different carcinomas of which 4 also included the primary tumour were obtained from the Department of Pathology Of these 19 cancer free liver tissue was obtained from 9 livers with metastases from carcinoma of the gastro intestinal tract Control tissues were obtained from 8 livers from patients who ... [illegible]

... failure Carcinoma and cancer free tissue from 10 kidneys were obtained at the De... [illegible]

Extracts and Eluates

Extracts and eluates were the same as used previously (16 17) having been stored at −25 °C The preparation has been described in detail elsewhere (16) In brief the tissue was homogenized in phosphate buffered saline pH 7 2 (PBS) (four times the wet weight) for 1 min in a Servall Omnimixer and centrifuged at 20 000 × g for 20 min The supernatant beneath the lipid layer was called the extract The tissue sediment was resuspended in PBS placed between glass fiber filters in a short glass column and washed in a continuous upward flow of PBS After washing the column was submerged in a water bath at 56 °C and the tissue eluted in the continuous flow of PBS at that temperature Two h was the usual time needed for elution of the tissue The eluates were concentrated to between 1 and 3 ml using Amicon Diaflo UM 05 filters The different proteins found in these eluates has been reported (14 16 17) The eluted tissue sediment was centrifuged at 20 000 × g for 20 min snap frozen in liquid nitrogen and mounted on specimen holders for the preparation of cryostat sections

Erythrocytes

Human OR_1R_2 erythrocytes (O) and sheep erythrocytes (E) were obtained and handled as previously described (15) In some experiments washed O and E were centrifuged at 6 000 × g for 10 min the pellet snap frozed in liquid nitrogen and then mounted on specimen holders for the preparation of cryostat sections Washed O and E were also treated with formaldehyde (7) and then lyophilized

Sera and Other Reagents

Samples of patient sera were from the same sera as were used previously (16 17) The human control serum used was from a pool of sera (PHS) from 20 blood donors Isolated IgG was purchased from AB Kabi Stockholm Antisera to human Ig were raised in rabbits by subcutaneous immunization using a mixture of isolated IgG in Freund's complete adjuvant Antiserum to E (A) was raised in rabbits by intravenous immunization FITC conjugated rabbit anti human Ig was purchased from DAKO immunoglobulins Copenhagen The specificity of the rabbit antisera was checked using the double diffusion test in agar Human anti D was kindly provided by the Blood Bank Haukeland Hospital Human sera containing IgG antibodies to heart muscle and sections of rat heart muscle were obtained from the Department of Microbiology When stated the sera were pepsin digested according to Stewart et al (12)

Protein A (pA) was isolated from *Staphylococcus aureus* (strain Cowan I) and was tested for activity according to Grov et al (2)

Bovine serum albumin (BSA fraction V) was purchased from Armour Pharmaceutical Company Eastbourne England

Mixed Haemagglutination (MA) Technique

Cryostat sections pretreated as stated under Results were incubated with eluates extracts or sera in a moist chamber at circa 20 °C for 60 min After washing in PBS they were incubated with the appropriate indicator cells for 30 min using the microculture slides (hanging drop slides) for the detection of Fcγ receptor activity (FcRA) as described previously (13) The indicator cells were

1) *With pA as bridge* A 1% suspension of E in PBS was incubated with a 1/4 agglutinating unit of A for 30 min at circa 20 °C (EA) (One agglutinating unit is defined as the amount of the highest dilution of A which agglutinates an equal amount of a 1% suspension of E) The EA were washed 3 times and were resuspended to a 1% suspension in PBS containing 1 µg pA/ml which was the lowest concentration of pA giving an optimal reaction The suspension was left for 30 min at circa 20 °C The cells were washed 3 times and resuspended to a 1% suspension in PBS containing 10 mg BSA/ml (PBS BSA) (EA pA)

2) *With anti-Ig as bridge* A 10% suspension of O was incubated with an excess of anti D at 37 °C for 20 min (OD) and washed 3 times in PBS A 1% suspension of OD in PBS was incubated for 30 min at 20 °C with pepsindigested rabbit anti human Ig diluted to 1/100th of the antiglobulin titre dilution This dilution gave the optimal reaction The cells were washed 3 times in PBS and resuspended to a 1% suspension in PBS BSA (OD anti Ig)

OD were also incubated with pA and used as indicator cells for MA However these indicator cells had a tendency to stick to the glass or to non reactive tissue The EA pA cells were therefore used

To check the reactivity of the indicator cells they were tested against selective tissues as shown in Table 1 EA pA detected only whole IgG bound to the sections whereas OD anti Ig detected both whole and $F(ab')_2$ of IgG

Immunofluorescence (IF) technique

Cryostat sections were pretreated as stated under Results They were then incubated with eluates extracts or sera for 60 min in a moist chamber and washed in PBS Afterwards they were incubated with FITC conjugated anti human Ig for 30 min at circa 20 °C washed in PBS and mounted in 50% glycerol in PBS The sections were examined using a Leitz Orthoplane Microscope equipped for epillumination with a Leitz vertical illuminator

To check the reactivity of the IF selected tissues were used as shown in Table 1 Both whole IgG and $F(ab')_2$ of IgG were detected

^{125}I pA

pA 200 µg was labelled with 2 mCi ^{125}I (Institute for Atomenergy Kjeller Norway) using the chloramin T method as described by Hunter (5) The free ^{125}I was separated from the ^{125}I pA using a Sephadex G 25 column The fractions containing ^{125}I pA were pooled and diluted to 20 ml (10 µg ^{125}I pA/ml) and stored in 0 1 ml volumes at −25 °C until used

Radioimmunoassay (RIA)

Tissue sediment eluted at 56 °C was lyophilized A triplicate of 5 mg of dried sediment was used for each

TABLE 1 *Mixed Haemagglutination and Indirect Immunofluorescence with Sections of Rat Heart or Human and Sheep Erythrocytes Incubated with Sera Containing IgG Antibodies or F(ab')₂ Antibodies*

Sections of incubated with	Mixed haemagglutination[a]		Immunofluorescence[b]
	EA pA[1]	OD anti Ig[2]	
Rat heart human IgG anti heart	256[c]	256	128
Rat heart F(ab')₂ of anti heart	<4	256	128
Human O ery anti D	512	512	256
Human O ery F(ab')₂ of anti D	<4	512	256
Sheep ery (E) rabbit IgG anti E	2048	<4	<4
Sheep ery (E) F(ab')₂ of anti E	<4	<4	<4

[a] Indicator cells [1] Sheep erythrocytes sensitized with rabbit immune IgG (EA) and pA (EA pA)
[2] Human O ery sensitized with human anti D (OD) and F(ab')₂ rabbit anti human Ig (OD anti Ig)
[b] FITC labelled anti human Ig
[c] Reciprocal of the highest dilution of antibody containing serum giving reaction

eluate tested To avoid non specific binding of proteins to the tissue the sediments were first resuspended in PBS containing 0 05% Tween 20 (PBS T₂₀) (1) and left for 20 min The tubes were then centrifuged for 5 min at 1 000 × g The sediment was resuspended in 0 2 ml of the corresponding eluate extract or serum The suspension was incubated for 60 min at circa 20 °C Afterwards the sediments were washed 3 times by centrifugation at 1 000 × g in PBS T20 and incubated with 0 1 µg ¹²⁵I pA in 0 3 ml of PBS T₂₀ for 60 min at circa 20 °C The sediments were then washed again 3 times and the activity counted in a gamma counter (Auto LOGIC Abbott Labs) The results were expressed as mean cpm of the triplicate samples The controls for the RIA were E sensitized with varying amounts of A O sensitized with an excess of anti D and 5 mg of lyophilized E or O incubated with varying amounts of A and anti D respectively

A RIA using tissue sections was tried but was not successful

RESULTS

Demonstration of Tissue Bound Ig

Ig was detected in washed tissue sediments of homogenized tissue using all 3 techniques (MA IF and RIA) and in washed sections of untreated tissue using MA and IF These sections were washed in PBS at 20 °C for 30 min further washing did not reduce the reaction with the sections Fluorescence was mainly located to the surface of the cells

Recombination of Eluted Ig to Tissue

Using eluted autologous tissue sediments no binding of the eluted Ig was obtained as revealed by Ig in the corresponding extracts or sera bind The lack of reactivity of Ig in eluates extracts and sera could however be due to destruction or elution of antigens during the 2 h elution procedure Sections of untreated malignant tissue were therefore eluted in PBS at 56 °C until no Ig could be detected using MA or IF The time needed was 30 min The eluted sections were then incubated with eluates extracts and sera The Ig bound to these sections If the sections were eluted for 2 h prior to incubation no binding was obtained The IF was difficult to read using eluted tissue sections due to non specific fluorescence whereas MA was easy to read Furthermore in titration experiments using different antisera (Table 1) the MA was slightly more sensitive than IF (1–2 titration steps) Therefore MA was used in the further studies and the results given were the mode or the median of three or more repeat experiments

The Ig eluted from normal tissues and from liver tissues from patients with non malignant diseases did not bind to the autologous tissue

Characterization of the Binding of Eluted Ig to the Tissue

Three eluates which bound strongly to the autologous tissue were pepsin digested Sections were incubated with the digested preparations and then tested in MA When EA pA was used as indicator cells no reaction was obtained whereas OD anti Ig gave the same reactions as seen with sections incubated with undigested eluates Accordingly the binding was mediated through the Fab portion of the Ig

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IgC

Extracts and Eluates

Extracts and eluates were the same as used previously (16 17) having been stored at −25 °C The preparation has been described in detail elsewhere (16) In brief the tissue was homogenized in phosphate buffered saline pH 7 2 (PBS) (four times the wet weight) for 1 min in a Servall Omnimixer and centrifuged at 20 000 × g for 20 min The supernatant beneath the lipid layer was called the extract The tissue sediment was resuspended in PBS placed between glass fiber filters in a short glass column and washed in a continuous upward flow of PBS After washing the column was submerged in a water bath at 56 °C and the tissue eluted in the continuous flow of PBS at that temperature Two h was the usual time needed for elution of the tissue The eluates were concentrated to between 1 and 3 ml using Amicon Diaflo UM 05 filters The different proteins found in these eluates has been reported (14 16 17) The eluted tissue sediment was centrifuged at 20 000 × g for 20 min snap frozen in liquid nitrogen and mounted on specimen holders for the preparation of cryostat sections

Erythrocytes

Human OR_1R_2 erythrocytes (O) and sheep erythrocytes (E) were obtained and handled as previously described (15) In some experiments washed O and E were centrifuged at 6 000 × g for 10 min the pellet snap frozed in liquid nitrogen and then mounted on specimen holders for the preparation of cryostat sections Washed O and E were also treated with formaldehyde (7) and then lyophilized

Sera and Other Reagents

Samples of patient sera were from the same sera as were used previously (16 17) The human control serum used was from a pool of sera (PHS) from 20 blood donors Isolated IgG was purchased from AB Kabi Stockholm Antisera to human Ig were raised in rabbits by subcutaneous immunization using a mixture of isolated IgG in Freund s complete adjuvant Antiserum to F (A) was raised in rabbits by intravenous immunization FITC conjugated rabbit anti human Ig was purchased from DAKO immunoglobulins Copenhagen The specificity of the rabbit antisera was checked using the double diffusion test in agar Human anti D was kindly provided by the Blood Bank Haukeland Hospital Human sera containing IgG antibodies to heart muscle and sections of rat heart muscle were obtained from the Department of Microbiology When stated the sera were pepsin digested according to *Stewart et al* (12)

Protein A (pA) was isolated from *Staphylococcus aureus* (strain Cowan I) and was tested for activity according to *Gros et al* (2)

Bovine serum albumin (BSA fraction V) was purchased from Armour Pharmaceutical Company Eastbourne England

Mixed Haemagglutination (MA) Technique

Cryostat sections pretreated as stated under Results were incubated with eluates extracts or sera in a moist chamber at circa 20 °C for 60 min After washing in PBS they were incubated with the appropriate indicator cells for 30 min using the microculture slides (hanging drop slides) for the detection of Fcγ receptor activity (FcRA) as described previously (13) The indicator cells were

1) *With pA as bridge* A 1% suspension of E in PBS was incubated with a 1/4 agglutinating unit of A for 30 min at circa 20 °C (EA) (One agglutinating unit is defined as the amount of the highest dilution of A which agglutinates an equal amount of a 1% suspension of E) The EA were washed 3 times and were resuspended to a 1% suspension in PBS containing 1 μg pA/ml which was the lowest concentration of pA giving an optimal reaction The suspension was left for 30 min at circa 20 °C The cells were washed 3 times and resuspended to a 1% suspension in PBS containing 10 mg BSA/ml (PBS BSA) (EA pA)

2) *With anti Ig as bridge* A 10% suspension of O was incubated with an excess of anti D at 37 °C for 20 min (OD) and washed 3 times in PBS A 1% suspension of OD in PBS was incubated for 30 min at 20 °C with pepsindigested rabbit anti human Ig diluted to 1/100th of the antiglobulin titre dilution This dilution gave the optimal reaction The cells were washed 3 times in PBS and resuspended to a 1% suspension in PBS BSA (OD anti Ig)

OD were also incubated with pA and used as indicator cells for MA However these indicator cells had a tendency to stick to the glass or to non reactive tissue The EA pA cells were therefore used

To check the reactivity of the indicator cells they were tested against selective tissues as shown in Table 1 EA pA detected only whole IgG bound to the sections whereas OD anti Ig detected both whole and $F(ab')_2$ of IgG

Immunofluorescence (IF) technique

Cryostat sections were pretreated as stated under Results They were then incubated with eluates extracts or sera for 60 min in a moist chamber and washed in PBS Afterwards they were incubated with FITC conjugated anti human Ig for 30 min at circa 20 °C washed in PBS and mounted in 50% glycerol in PBS The sections were examined using a Leitz Orthoplane Microscope equipped for epillumination with a Leitz vertical illuminator

To check the reactivity of the IF selected tissues were used as shown in Table 1 Both whole IgG and $F(ab')_2$ of IgG were detected

^{125}I pA

pA 200 μg was labelled with 2 mCi ^{125}I (Institute for Atomenergy Kjeller Norway) using the chloramin T method as described by *Hunter* (5) The free ^{125}I was separated from the ^{125}I pA using a Sephadex G 25 column The fractions containing ^{125}I pA were pooled and diluted to 20 ml (10 μg ^{125}I pA/ml) and stored in 0 1 ml volumes at −25 °C until used

Radioimmunoassay (RIA)

Tissue sediment eluted at 56 °C was lyophilized A triplicate of 5 mg of dried sediment was used for each

TABLE 3 *Mixed Haemagglutinati n^a) with Secti ns of Carcinoma and Cancer free Tissue from the same Organ The Sections Were Eluted at 56 °C f r 30 min pri r to Incubat on with the Eluates*

Tissue	Incubated with eluates of	No of tumours giving		
		Similar reaction^b)	Weaker reaction	No reaction
Carcinoma of the kidney	Carcinoma of the kidney	4	4	2
Carcinoma of the kidney	Cancer free renal tissue	2	6	2
Cancer free renal tissue	Carcinoma of the kidney	1	6	3
Cancer free renal tissue	Cancer free renal tissue	2	5	3
Liver metastases	Liver metastases	3	4	2
Liver metastases	Cancer free liver tissue	1	3	5
Cancer free liver tissue	Liver metastases	0	6	3
Cancer free liver tissue	Cancer free liver tissue	0	5	4

a) Indicator cells OD anti Ig (see Table 1)
b) Reaction compared to non-eluted tissue sections (see text)

TABLE 4 *Mixed Haemagglut nat on with Sect ons of Carcinomas of the Kidney Incubated with Eluates of the Tissue^a)*

Eluates from carcinoma No	Tissue from carcinoma No			
	1	2	3	4
1	+	−	+	+
2	+	+	+	+
3		+	+	+
4	+	−	+	+

a) Indicator cells OD anti Ig (see Table 1)
+ Binding of indicator cells
No binding of indicator cells

the gastro intestinal tract also bound to sections of some carcinoma of the kidney The results obtained sho ed ...

... as well as reactivity for cancer free tissue from other individu als

DISCUSSION

The purpose of the present study was to investigate whether Ig eluted from human carcinomas could recombine to the tissue One approach was to use the tissue sediment from which the Ig was eluted No binding of the eluted Ig to eluted autologous tissue sediment was however obtained Nor did the Ig in extracts or sera bind The lack of binding of th s Ig ...

... the antigens Therefore sections eluted for 30 min were used Since this treatment also abolishes the FcRA (13) FcR should not interfere with the binding of Ig

MA appeared most suitable for the detection of tissue bound Ig pA was useful as a bridge for the indicator cells This system was as sensitive as the system using anti Ig as a bridge This is in line with the results obtained by *Portanova et al* (10) who studied IgG antibodies bound to cell surface antigens on cells in suspension In our experiments IF was not found useful since non-specific fluore scence occurred after elution of the tissue Nor was RIA useful since eluted tissue sediments could not be used (see above) and no technique using RIA with tissue sect ons ...

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systems used to study the binding of eluted Ig it could be concluded that most of the bound Ig was of ...

... cinoma of the lung and sarcoma respectively They found by applying IF with monospecific antisera that the eluted IgG bound to

250 mg/l) No binding of this IgG was obtained Incubation of the sections with 20 mg BSA/ml in PBS prior to the incubation with the eluates did not inhibit the binding of the eluted Ig to the sections The results obtained therefore indicate that eluates contained Ig antibodies to the tissue

Binding of Eluted Ig to Autologous Tissue

Ig eluted from 21 tumours bound to the autologous tissue, whereas the eluted Ig from the other 8 did not bind Similar results were obtained using the corresponding extracts or sera

To evaluate how much of the Ig in each eluate bound to the tissue, the reaction on sections incubated with the eluates were compared with the reaction on non-eluted tissue sections (washed tissue sections, see above) Sections incubated with the eluates using 9 of the tumours, revealed a reaction similar to the non-eluted sections, whereas eluates of the other 12 showed a weaker reaction All eluates tested showed similar results using both indicator systems

We have previously shown (14, 17) that the ratio of albumin to IgG in the eluates and extracts were similar using 5 of the tumours, indicating a predominance of non-specifically bound IgG None of these eluates bound to the tissue Eluates of 2 of the tumours contained marker antibodies for non-specifically bound IgG, indicating both specifically and non-specifically bound IgG (16) These 2 eluates gave a weaker binding of eluted Ig

Eluates of metastases gave similar reactions, when incubated on the autologous tissue and on the corresponding primary tumours Eluates of these primary tumours were not available for testing

Binding of Eluted Ig to Different Tissues

Eluates of carcinoma and cancer-free tissue from the same organ were incubated on the sections of both types of tissue The example given in Table 2 shows that Ig eluted from the carcinomas bound to both the cancer and the cancer-free tissue The amount of Ig bound to the cancer tissue was apparently the same as the amount in the non eluted tissue, whereas less Ig bound to the cancer free tissue The Ig eluted from the cancer-free tissue also bound to both types of tissue, but less Ig bound to these tissues than that detected in non eluted tissue The results obtained using all eluates of carcinomas and cancer-free tissue from the same organ are shown in Table 3 Fischer exact test on a 2×2 table for cancer tissue and eluates of cancer against the other combinations with weaker reaction and no reaction combined gave $p < 0.02$ Accordingly, significantly more of the Ig eluted from the cancers bound to the autologous tissue than the amounts bound when other combinations were tested

Eluates of 4 different carcinomas of the kidney were also incubated on sections of the different tissues (Table 4) Ig eluted from all of the tumours bound to 2 of the tumours Ig eluted from 1 tumour bound to all of the tissues Similar results were also obtained using eluates of cancer-free tissue The Ig in eluates of some liver metastases of carcinoma of

TABLE 2 *Mixed Haemagglutination with Sections of a Carcinoma of the Kidney and the Cancer-free Tissue from the same Organ Incubated with the Corresponding Eluates*

Pre treatment of tissue sections	Incubated with	Sections of			
		Carcinoma		Cancer free tissue	
		a) EA pA	OD anti Ig	EA pA	OD anti Ig
Washed at 20 °C for 30 min	PBS	++	++	++	++
Eluted at 56 °C for 30 min	PBS	–	–	–	–
Eluted at 56 °C for 30 min	Eluate of carcinoma	++	++	+	+
Eluted at 56 °C for 30 min	Eluate of cancer free tissue	+	+	+	+

a) Ind[illegible] See Table I [illegible] sections
++
+
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TABLE 3 *Mixed Haemagglutination[a]) with Sections of Carcinoma and Cancer free Tissue from the same Organ The Sections Were Eluted at 56 °C for 30 min prior to Incubation with the Eluates*

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[a]) Indicator cells OD anti Ig (see Table 1)
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	1	2	3	4
1	+	−	+	+
2	+	+	+	+
3		+	+	+
4	+		+	+

[a]) Indicator cells OD anti Ig (see Table 1)
\+ Binding of indicator cells
− No binding of indicator cells

the gastro intestinal tract also bound to sections of some carcinoma of the kidney The results obtained showed that the Ig eluted from the carcinoma had reactivity for tissues from other carcinomas of both the same type and of other types as well as reactivity for cancer free tissue from other individuals

DISCUSSION

The purpose of the present study was to investigate whether Ig eluted from human carcinomas could recombine to the tissue One approach was to use the tissue sediment from which the Ig was eluted No binding of the eluted Ig to eluted autologous tissue sediment was however obtained Nor did the Ig in extracts or sera bind The lack of binding of this Ig in eluates extracts and sera was shown to be due to elution or destruction of antigens during the 2 h elution procedure Elution of tissue sections for 30 min in PBS at 56 °C did not seem to influence the antigens Therefore sections eluted for 30 min were used Since this treatment also abolishes the FcRA (13) FcR should not interfere with the binding of Ig

MA appeared most suitable for the detection of tissue bound Ig pA was useful as a bridge for the indicator cells This system was as sensitive as the system using anti Ig as a bridge This is in line with the results obtained by *Portanova et al* (10) who studied IgG antibodies bound to cell surface antigens on cells in suspension In our experiments IF was not found useful since non specific fluorescence occurred after elution of the tissue Nor was RIA useful since eluted tissue sediments could not be used (see above) and no tech

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systems used to study the binding of eluted Ig it could be concluded that most of the bound Ig was of the IgG class with an intact Fc-portion The results obtained accord with those obtained by *Ioachim et al* (6) and *Romsdahl & Cox* (11) who used acid eluates of carcinoma of the lung and sarcoma respectively They found by applying IF with monospecific antisera that the eluted IgG bound to

250 mg/l) No binding of this IgG was obtained Incubation of the sections with 20 mg BSA/ml in PBS prior to the incubation with the eluates did not inhibit the binding of the eluted Ig to the sections The results obtained therefore indicate that eluates contained Ig antibodies to the tissue

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		Carcinoma		Cancer free tissue	
		a) EA pA	OD anti Ig	EA pA	OD anti Ig
Washed at 20 °C for 30 min	PBS	++	++	++	++
Eluted at 56 °C for 30 min	PBS	–	–	–	–
Eluted at 56 °C for 30 min	Eluate of carcinoma	++	++	+	+
Eluted at 56 °C for 30 min	Eluate of cancer free tissue	+	+	+	+

a) Indicator cells See Table 1
++ Tight monolayer of indicator cells attached to the sections
+ Single indicator cells attached to the sections
– No indicator cells attached to the sections

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cells in suspension of the autologous tumours In addition, *Romsdahl & Cox* (11) showed that eluted IgA bound to the sarcoma cells *Gupta & Morton* (3) eluted Ig from melanoma tissue using 15% NaCl at 37 °C and found evidence for *in vivo* binding of eluted IgG using a complement fixation test

In the present study the binding of the eluted IgG to the sections was considered to be mediated through the Fab portion since a) pepsin digested eluted IgG bound to the tissue, and b) BSA did not inhibit the binding of eluted IgG

The IgG eluted from the carcinomas also bound to the cancer-free tissue from the same organ from the same patients This is in contrast to the results obtained by *Romsdahl & Cox* (11) *Gupta & Morton* (3) and *Ioachim et al* (6) who found no binding to different control tissues of IgG eluted from malignant tissue These differences can be due to different techniques used since *Romsdahl & Cox* (11) and *Ioachim et al* (6) used cells in suspension and *Gupta & Morton* (3) used complement fixation test with sonically treated tissue residue

In the present study IgG eluted from one carcinoma of the kidney bound to other carcinomas of the kidney and IgG eluted from carcinomas of the gastro intestinal tract also bound to the carcinoma of the kidney This is in line with the results reported by *Gupta & Morton* (3) that eluted IgG from melanoma bound to tissues from other melanomas

The results obtained here show that IgG eluted from human carcinoma has a broad tissue reactivity The results obtained were not unexpected since the same activity was found in the corresponding extracts and sera These results accord with those previously observed that sera from patients with cancer contain in addition to specific tumour antibodies antibodies to other tumours as well as to different normal tissues (for review see ref 8) Increased amounts of auto antibodies are also found in sera of cancer patients (9)

The IgG eluted from the cancer free tissue in this study also bound to the cancer tissue from the same organ showing that the malignant tissue has some of the antigens found in the cancer free tissue

Measurement of the amount of IgG in tissue using MA with tissue sections is not accurate However since RIA could not be used the only way to estimate how much of the eluted IgG bound to the eluted tissue, was to compare the strength of the reaction using eluted sections incubated with the eluate to the reaction using non eluted sections Apparently most of the eluted IgG from 9 of the tumours seemed to bind to the tissue whereas only parts of the eluted IgG from the other 12 tumours bound This indicates that parts of the eluted IgG from these tumours could be non specifically bound or bound to receptors such as FcR At present there is no technique available to distinguish between these types of binding

The IgG eluted from 8 tumours did not bind to the autologous tissue The IgG eluted from 5 of these tumours was considered non specifically bound since the ratio of albumin to IgG was similar using extracts and eluates from these tumours (14 17) The other 3 tumours displayed a reduced ratio of albumin to IgG in the eluates compared to the corresponding extract (14 17) This IgG could be bound to FcR Another possibility is that antigens eluted concomitantly with the IgG block the binding But due to the lack of eluates no investigations were performed to clarify this point

The IgG eluted from different normal tissues and from liver tissues from patients with various non malignant diseases did not bind to the autologous tissue Accordingly, the Ig associated with these tissues were non specifically bound or bound to receptors such as FcR

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